REMARKS

The Applicants thank the Office for the examination to date and for finding claims 51, 54, and 56 allowable if they are written in independent form. The Applicants respectfully request reconsideration of the present application.

I. Status of the Claims

New claims 57-59 are added to recite specific embodiments recited in independent claim 45. Particularly, claims 57 and 58 separately recite specific antibody embodiments, and claim 59 further recites the specific humanized antibody embodiments recited in claim 53. No new matter is introduced, and claims 45-49 and 51-59 are currently pending to be examined on their merits.

II. Claim Rejection – 35 U.S.C. § 102

Claims 45-49, 52-53, and 55 are rejected under 35 U.S.C. § 102(e) as allegedly being anticipated by US 5,986,065 ("Wong"). The Applicants respectfully traverse the rejection.

As stated in the June 4, 2009 Office Action and the subsequent September 25, 2009 Advisory Action, the Office's main concerns are (1) whether Wong's teaching regarding suppressing "hypertrophy of the vascular intima" is inherent; (2) whether "hypertrophy of the vascular intima" can be equated with "restenosis"; (3) whether one of ordinary skill in the art would appreciate the differences between the different stages of "restenosis"; and (4) whether Wong teaches more than thrombosis. *See* Advisory Action. The Applicants respectfully traverse the Office's assertions with respect to these concerns.

(i) The Office's interpretation of the present claims is incorrect

In the second paragraph in the Advisory Action, the Office asserts that the Office has not relied on inherency and its position is based on Wong's specific teaching with respect to the "same antibodies recited in the instant claims." However, in the last paragraph, the Office alleges that "since the antibodies of Wong et al. have the same activity as those instantly claimed,

they **inherently** have the same effect upon restenosis as the claimed antibodies." (emphasis added). The Applicants respectfully submit that the Office's position appears inconsistent.

Regardless of whether the Office relies on inherency, the Applicants respectfully submit that no such inherency exists. The Office's allegation is based on the **antibodies** of Wong and the presently claimed invention. However, both present independent claims 45 are 53 are explicitly directed to **methods** of **suppressing hypertrophy** specifically for **a patient in need thereof**. Thus, the Office's reliance on the antibodies of Wong to reject the presently claimed methods (items (1) and (4), Advisory Action), particularly when the presently claimed methods are directed to a specific treatment, is erroneous. The Office's incorrect claim interpretation is further evidenced in the assertion in the last sentence in the Advisory Action "...the patient group 'in need thereof' to be treated by the instant claims and by Wong et al. would appear to be the same." The Applicants respectfully submit that it matters little what the antibody of Wong is. Because the present claims are directed to specific type of treatment **methods** for a specific type of a patient, the present claims should be interpreted accordingly.

The Office incorrectly interprets the present claims to encompass more than suppressing hypertrophy for a patient in need thereof, as specifically recited in the present claims. This interpretation is in direct conflict to the Federal Circuit case law, as provided in the September 3, 2009 reply. *See Jansen v. Rexall Sundown*, 342 F.3d 1329 (Fed. Cir. 2003), *Rapoport v. Dement*, 254 F.3d 1053 (Fed. Cir. 2001). To reiterate, the *Rapoport* holding was summarized as follows in *Jansen v. Rexall Sundown*, 342 F.3d 1329 (Fed. Cir. 2003):

> A similar issue arose in *Rapoport,* an interference proceeding before the PTO's Board of Patent Appeals and Interferences. The count in that case read as follows:
>
> > A method *for treatment of sleep apneas* comprising administration of a therapeutically effective amount of a Formula I azapirone compound or a pharmaceutically effective acid addition salt thereof *to a patient **in need of** such treatment* ....

> 254 F.3d at 1056 (emphases added). On appeal we gave weight to the ordinary meaning of the preamble phrase "for treatment of sleep apneas," interpreting it to refer to sleep apnea, ***per se,*** **not just "symptoms associated with sleep apnea**." *Id.* at 1059. Rapoport argued that the count was unpatentable on the ground that a prior art reference disclosed that a form of the compound recited in the claim could be administered, not for treatment of sleep apnea itself, but for treatment of anxiety and breathing difficulty, a symptom of apnea. *Id.* at 1061. We rejected that argument, stating, "There is no disclosure in the [prior art reference that the compound] is administered to patients suffering from sleep apnea ***with the intent to cure the underlying condition.***" *Id.* (emphasis added). Thus, the claim was interpreted to require that the method be practiced with the intent to achieve the objective stated in the preamble.

Jansen 342 F.3d 1329. (Bold emphasis added). It is clear that *Jansen* and *Rapoport* apply to the present claims.

Similar to *Rapoport,* the present claims are intended to recite a method of suppressing hypertrophy of the vascular intima for a patient **in need thereof**, and thus they should analogously be interpreted to suppress "hypertrophy of the vascular intima," *per se*, and not merely "symptoms associated" therewith. Wong discloses only the prevention of thrombosis and blood clotting and does not at all teach the presently claimed methods of suppressing "hypertrophy of the vascular intima caused by tissue expression" in a patient "in need thereof," as recited in independent claims 45 and 53. Accordingly, the Office's inclusion of "anti-coagulant activity" of Wong in its interpretation of the recitation "suppressing hypertrophy of the vascular intima" in the present claims is erroneous.

*(ii) Hypertrophy of vascular intima is **not** thrombosis*

The thrombosis prevention of Wong's teaching is distinct from the presently claimed hypertrophy suppression. Despite the fact that Wong mentions the term "restenosis," Wong's teaching in its entirety is related only to anticoagulant activity and has little to do with suppressing hypertrophy. The distinctions and the Office's incorrect conclusion with respect to

"restenosis" is explained further below. The Office also extrapolates and then alleges that the presently claimed hypertrophy "would encompass both early and late stages of restenosis" (item (4), Advisory Action). The Applicants respectfully submit that the Office's reliance on a general descriptive term "restenosis" to produce its own definition for Wong's teaching and the presently claimed invention is unreasonable, especially when the Office's definition is contradictory to commonly understood knowledge.

The Office asserts that because the present Specification does not provide a specific definition of the term "restenosis," the Office provides its own definition. The Applicants respectfully submit that the term "restenosis" is known in the art and respectfully remind the Office that a patent need not teach, and preferably omits, what is well known in the art *See, e.g., Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986). In the September 3, 2009 reply, the Applicants submitted a specific reference to a chapter in Fuster *et al.* to demonstrate that the term restenosis is a general term that involves a series of distinct events, whereas Wong's teaching relates only to one stage that is distinct from what is presently claimed. Nevertheless, the Office alleges that the submission of such a reference is at best an "assertion." See item (3), Advisory Action.

The Applicants respectfully submit that the Office's analogy between an "argument of counsel" and a specific reference to "factual evidence" is unreasonable. However, to facilitate the Office's review, the Applicants submit herewith Exhibit A, which contains a copy of the aforementioned chapter; the section referenced in the previous reply is also explicitly marked. The Applicants also submit herewith an *Ann Thorac Surg* article by Thatte *et al.* as Exhibit B to provide additional support and respectfully direct the Office's attention to Fig. 1 and the description associated therewith in Exhibit B.

Exhibits A and B together demonstrate that while each of the different events during restenosis might occasionally be mistakenly labeled as "restenosis," restenosis is understood by one of ordinary skill in the art to involve a series of distinct events. Thus, although Wong might have mentioned the term "restenosis," Wong's teaching in its entirety is directed to anti-

coagulant activity to prevent thrombosis and has little to do with suppressing the **hypertrophy** that occurs **after** thrombosis. The Office repeatedly directs the Applicants to col. 3, lines 18-37 of Wong, which as a matter of fact supports the Applicants' position. Col. 3, lines 18-37 of Wong reads "...alleviate various thromboses, particularly to prevent or inhibit restenosis, or other thromboses following an invasive medical procedure..." Both the plain language of this statement and *ejusdem generis* suggest that "restenosis" in the clause "particularly..." should be read together with and to refer to the "thromboses" in the phrase "various thromboses" immediately before and the phrase "other thromboses" immediately after. In fact, Wong does not contain the term "hypertrophy" at all in its disclosure. Accordingly, the Office has misconstrued Wong's teaching. Furthermore, in view of the foregoing, the Office's extrapolation from "hypertrophy of the intima," as recited in the present claims, to "thrombosis prevention" of Wong based solely on the fact that the general term "restenosis" is mentioned in Wong and Example 6 of the present Specification (see item (2), Advisory Action) is arbitrary and without basis.

Finally, the Office appears to suggest that the Applicants have admitted that hypertrophy of vascular intima is restenosis, referring to page 7 of the December 1, 2008 reply. This is simply untrue. The Applicants respectfully invite the Office to read the remaining paragraph following the sentence pointed to by the Office. In fact, the sentence immediately after reads "there are **two** major mechanisms to distinguish for restenosis. The **first one is thrombosis**.... The **second form of restenosis is tissue growth** .... is not prevented by anti-clotting formation." Thus, the Applicants respectfully traverse the Office's reading of that paragraph and the Office's taking of one sentence from a paragraph out of context and the Office's conclusion drawn therefrom.

Thus, in view of Exhibits A and B and the explanation above, particularly with respect to the Federal Circuit case law, the presently claimed methods should be read as relating to treatments of a hypertrophy of vascular intima in a patient need thereof. The hypertrophy is an event distinct from the thrombosis as taught by Wong. Because Wong does not teach each and

every element recited in present independent claims 45 and 53, Wong's teaching cannot anticipate these claims or their corresponding dependent claims.

Therefore, at least in view of the foregoing, the Applicants respectfully request withdrawal of the rejection.

CONCLUSION

The Applicants believe that the present application is now in condition for allowance and respectfully request favorable reconsideration of the application.

The Office is invited to contact the undersigned by telephone if a telephone interview would advance the prosecution of the present application.

The Office is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicants hereby petition for such extension under 37 C.F.R. § 1.136 and authorize payment of any such extensions fees to Deposit Account No. 19-0741.

Respectfully submitted,

Date Nov. 16, 2009

By _____

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EXHIBIT A

CHAPTER 7

Kathy K. Griendling / David G. Harrison / R. Wayne Alexander

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It has become apparent that a diverse number of pathologic processes contribute to common vascular diseases, such as atherosclerosis and hypertension. During the past several years, these pathologic events have been defined with increasing clarity at a cellular and molecular level, and strategies are emerging to treat these primary processes rather than simply treating the secondary manifestations of vascular disease. Because of this, an understanding of the normal functions of vascular cells and how they are altered by various vascular insults has become essential for both basic investigators and clinicians caring for patients with peripheral vascular disease coronary artery disease, and hypertension. This chapter introduces important concepts in vascular biology and emphasizes how fundamental aspects of vascular control are altered by common disease conditions.

THE ENDOTHELIAL CELL

Normal endothelial cell function is crucial to homeostasis in the vascular system. During the past 15 years, it has become apparent that diseases such as atherosclerosis are ultimately manifestations of endothelial dysfunction. Normally, the endothelium has three major roles: (1) it is a metabolically active secretory tissue; (2) it serves as an anticoagulant, antithrombotic surface; and (3) it provides a barrier to the indiscriminate passage of blood constituents into the arterial wall. The implications of these physiologic properties for vascular biology are considered separately.

Endothelial Cell Metabolism and Secretion of Vasoactive Factors

As discussed in more detail below, endothelial cells secrete vasoactive substances that play a major role in the control of vascular tone. These molecules include vasodilators such as prostacyclin, endothelial-derived relaxing factor (EDRF), and endothelial-derived hyperpolarizing factor (EDHF).[1–3] In addition, the endothelium produces vasoconstrictor substances, including endothelin[4] and vasoconstrictor prostanoids.[5]

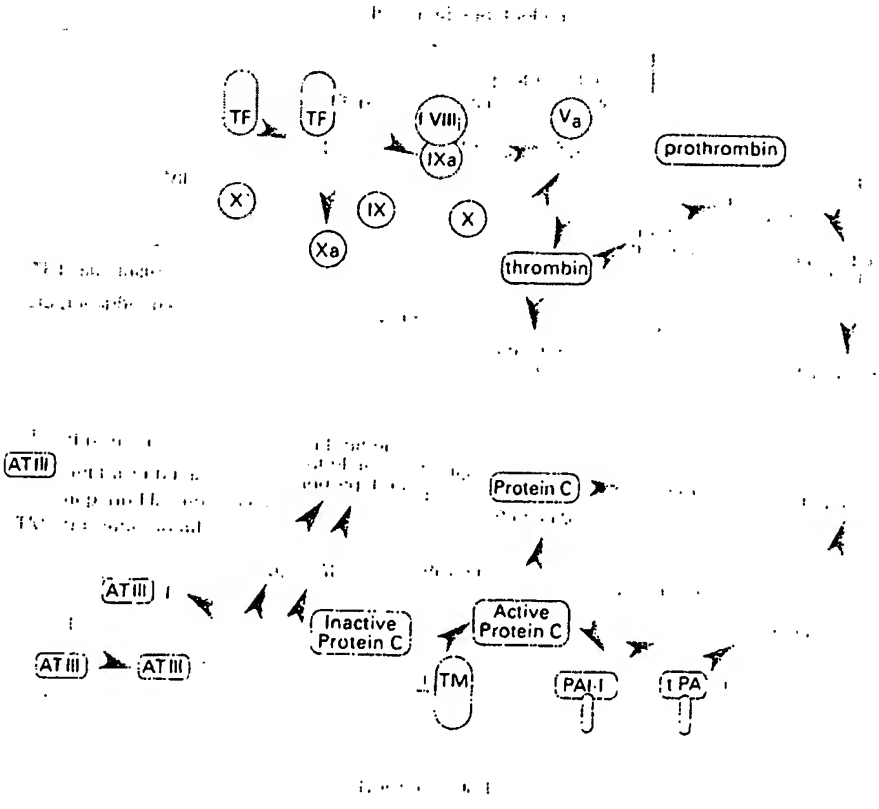
Endothelial cells also manufacture and secrete substances such as factor VIII antigen, von Willebrand's factor, tissue factor, thrombomodulin, and tissue plasminogen activator, which are all involved in coagulation/fibrinolytic pathways. Structural components of the extracellular matrix synthesized by these cells include collagen, elastin, glycosaminoglycans, and fibronectin.[6,7] The composition of the extracellular matrix is dynamically modulated by matrix metalloproteinases, enzymes that degrade matrix protein and participate in its remodeling. These enzymes are secreted by both endothelial and smooth muscle cells.[8,9] In addition, endothelial cells synthesize and secrete heparans and growth factors that regulate smooth muscle cell proliferation.[10–13] Finally, endothelial cells are able to clear and metabolically alter bloodborne and locally produced substances, including plasma lipids and lipoproteins,[14] adenine nucleotides and nucleosides,[15] serotonin, catecholamines, bradykinin, and angiotensin I.[16]

Endothelial cells are involved in the metabolism of plasma lipids in several ways. Lipoprotein lipase, an enzyme that hydrolyzes triglycerides into constituent fatty acids, is bound to the endothelial cell surface by heparan sulfates.[17] The interaction of this enzyme with chylomicrons or very low density lipoprotein (VLDL) particles results in the release of free fatty acids, which can then cross the subendothelial space to the underlying smooth muscle or inflammatory cells in atherosclerosis. In addition, endothelial cells possess receptors for low-density lipoprotein (LDL),[18] which regulate the transport and modification of LDL. Normally, LDL receptors are downregulated because receptor processing is inhibited in the nonproliferating monolayer.[18] There are, however, two other pathways for uptake of LDL. First, LDL can be transported across the endothelium by an active process that is likely independent of plasmalemmal vesicles but may utilize paracellular gaps or fixed transendothelial channels.[19] Second, modified, or oxidized LDL

can be taken up by "scavenger" LDL receptors,[20] which include SRA, SR-BI, CD36, and the lectin-like oxidized LDL receptor 1 (LOX-1).[21,22] Endothelial cells also have the capacity to modify LDL,[23] thus enhancing its uptake, ultimately leading to an increase in cholesterol esters in the vessel wall.

The Endothelial Cell and Thrombosis

Quiescent endothelial cells normally present an antithrombotic surface that inhibits platelet adhesion and coagulation. (For a more detailed discussion of thrombosis, see Chap. 44.) Endothelial cells are, however, capable of synthesizing and secreting prothrombotic factors, especially when stimulated with cytokines or other inflammatory agents. The endothelium thus represents a functional antithrombotic-thrombolytic/thrombotic balance. Potent anticoagulants elaborated by the endothelium include prostacyclin and nitric oxide, which inhibit platelet aggregation[24]; antithrombin III[25]; heparin-like molecules[26]; thrombomodulin, which activates protein C and is also expressed by the endothelium[27]; and tissue plasminogen activator (t-PA). Procoagulant factors that can be produced by the endothelium include tissue factor,[28] factor VIII, factor Va, and PAI-1 (Fig. 7-1). Conditions of injury or inflammation enhance the prothrombotic state of the endothelium by stimulating production of tissue factor and PAI-1. There has been considerable interest in the role of PAI-1 in vascular disease. PAI-1 levels are substantially elevated in humans with atherosclerosis and even higher in the setting of acute coronary syndromes. Moreover, the metabolic syndrome—consisting of dyslipidemia, obesity, and insulin resistance—is associated with higher levels of PAI-1. Angiotensin II and thrombin likewise stimulate endothelial PAI-1 production, promoting thrombosis.[29] Thus, under inflammatory conditions, endothelial cells can amplify the prothrombotic response. Not all of the factors controlling the expression of pro- and antithrombotic/fibrinolytic molecules are known, but it is clear that the endothelium functions as a major regulator of hemostasis.



Pathways of thrombosis and thrombolysis. Under normal conditions, the endothelium is antithrombotic. Antithrombin III (ATIII) binds thrombin and serves to clear thrombin from the circulation. Prostacyclin (prostaglandin I_2, PGI_2) inhibits platelet aggregation, and thrombomodulin (TM) activates protein C, which inhibits plasminogen activator inhibitor I (PAI-I) and interacts with protein S to inactivate activated factors V and VIII, thus limiting thrombosis. Since PAI-I inhibits the tissue plasminogen activator (t-PA)–catalyzed conversion of plasminogen to plasmin, PAI-I inhibition leads to accumulation of plasmin and fibrinolysis. Upon stimulation with inflammatory cytokines, there is increased expression of tissue factor on the endothelial cell surface. Tissue factor participates in the activation of factor X, which, in turn, promotes assembly of the prothrombinase complex, producing thrombin. Under these conditions, endothelial cells thus amplify the thrombotic response. (Courtesy of Bernard Lassegue, Ph.D.)

Endothelial Cell Permeability

A very important role of the endothelium is regulation of permeability to macromolecules. The consequences of fluid and macromolecular transport vary depending on vessel size. In large vessels, these processes contribute to vessel nutrition and act as a selective barrier. In the microcirculation, endothelial permeability regulates delivery of nutrients to target organs and exchange of metabolic by-products.

The major two mechanisms regulating endothelial barrier function involve modulation of intercellular contacts and transendothelial vesicular transport in caveolae. Two types of junctions regulate endothelial cell contact: adherin junctions and tight junctions. Adherin junctions contain the protein VE-cadherin, which is essential for maintenance of inter-endothelial cell contacts. VE-cadherin associates with catenins, plakoglobulin, and the actin cytoskeleton to support cell adhesion. Tight junctions are composed of occludins, claudins, and junctional adhesion molecule-1 (JAM-1). Regulation of these inter-endothelial cell contacts is dynamic and important in modulation of new vessel growth, the extravasation of leukocytes, and macromolecule leakage. The nature of these intercellular contacts varies substantially depending on the vessel size and location. For example, tight junctions are well developed in the blood-brain barrier but are less structurally defined in postcapillary venules, where fluid and solute transport is active. Capillaries and postcapillary venules respond to vasoactive agents—including the vascular endothelial cell growth factor (VEGF), histamine, and prostaglandins—with increased flux through these sites.[30] The tight junctions found in arteries tend to be more occlusive but may also be influenced by various agonists. Dynamic regulation of these pathways enables the endothelium to serve as a selective barrier, modulating access of highly mitogenic, thrombotic, or vasoactive substances to the underlying vascular smooth muscle.

Transendothelial vesicular transport is mainly utilized by the cell to transfer water-soluble macromolecules from the luminal surface to the abluminal surface. It has recently been shown that caveolae, vesicles containing the structural protein caveolin that are pinched off from the plasma membrane, are involved in transendothelial transport of macromolecules.[31,32] Caveolae are also sites where a variety of kinases, docking proteins, G proteins, and receptors reside[33] and therefore play an extremely important role in endothelial cell signal transduction.

Another major mechanism modulating endothelial barrier formation is endothelial cell contraction, analogous to smooth muscle contraction. This occurs in response to a variety of agonists, including thrombin, histamine, and ionomycin, and results in changes in cell shape that open gap junctions between cells. It is likely that this contractile response is a major mechanism for edema formation in response to histamine and bradykinin and is also involved in solute transport. This phenomenon is mediated by a series of intracellular signaling events, including activation of protein kinase C, myosin light-chain phosphorylation, activation of tyrosine kinases, and stimulation of the small G protein Rho.[34–36]

Thus, the endothelium has both passive and active roles in the control of vascular permeability by acting as a physical permeability barrier and by modulating the expression of cell surface and secreted agonists and molecules that are capable of altering permeability.

Endothelial Control of Vascular Tone

The endothelium serves a dual function in the control of vascular tone (Fig. 7-2). It secretes relaxing factors such as nitric oxide, prostacyclin, and the endothelium-derived hyperpolarizing factor as well as constricting factors such as endothelin. Vessel tone thus depends on the balance between these factors as well as on the ability of the smooth muscle cell to respond to them. The most important regulatory molecules are discussed separately.

NITRIC OXIDE

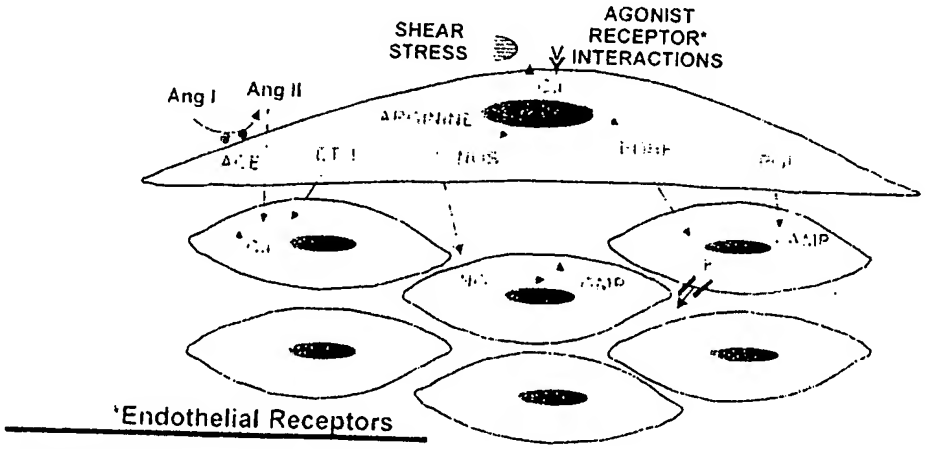
An endothelium-derived relaxing factor, (EDRF) was first described by Furchgott and Zawadzki,[2] who observed that aortic rings dilated in response to acetylcholine only when the rings maintained an intact endothelium. The EDRF was subsequently found to be nitric oxide (NO).[37]

NO is produced by the action of the enzyme NOS, which oxidizes the guanidino nitrogens of L-arginine to form citrulline and NO. This enzyme has been cloned from brain (nNOS, for neuronal NOS, type I),[38] macrophages (iNOS, for inducible NOS, type II),[39] and endothelial cells (eNOS, for endothelial NOS, type III).[40] The three isoforms of NOS share important consensus sequences for NADPH, flavin adenine dinucleotide, and flavin mononucleotide cofactor-binding sites, as well as a Ca^{2+}-calmodulin–binding site. An important cofactor for the NO synthases is tetrahydrobiopterin, which participates in electron transfer from the heme group of the enzyme to L-arginine. Interestingly, when tetrahydrobiopterin or L-arginine is absent, electron transfer is shunted to molecular oxygen, resulting in formation of the superoxide anion.[41] This phenomenon has been termed *uncoupling* of NOS, and there are substantial data that this may occur in a variety of disease states.[42]

Many factors have been shown to regulate the release of NO.[43] These include hormones such as acetylcholine, norepinephrine, bradykinin, thrombin, ATP, and vasopressin; the platelet-derived factors, serotonin and histamine; fatty acids; ionophores; and shear stress. NO easily crosses the smooth muscle cell membrane and binds to the heme moiety of the soluble guanylate cyclase, thereby enhancing the formation of cyclic GMP. Cyclic GMP, in turn, reduces intracellular Ca^{2+} concentrations, leading to dephosphorylation of the myosin light chain and relaxation.[44] It should be noted that the drug nitroglycerin exerts its vasodilator effects by being converted to NO, thus substituting for a natural product.

Although increases in intracellular calcium in response to the above agents clearly activate eNOS via binding of calcium/calmodulin, phosphorylation of the enzyme is important in regulating its activity. For example, shear stress acutely stimulates the release of NO from the endothelium, and this depends only on calcium during the first few seconds of the response.[45] The continued activation of eNOS in response to several minutes or hours of shear is maintained by serine phosphorylation.[46]

While expression of the endothelial enzyme (eNOS) was originally thought to be constitutive, it is now clear that its expression is highly regulated. Increases in shear stress rather markedly enhance expression of eNOS.[47] Likewise, low shear is associated with a decrease in eNOS expression. Exercise training dramatically increases



Endothelial control of vascular tone. Endothelial cells synthesize and secrete both vasodilator substances (NO, EDHF, and PGI_2) and vasoconstrictor compounds (Ang II and ET-1). Secretion of these factors occurs in response to receptor stimulation and hemodynamic forces such as shear stress. Vessel tone depends on the balance between these factors as well as on the ability of the smooth muscle cells to respond to them. NO = nitric oxide; NOS = nitric oxide synthase; EDHF = endothelial-derived hyperpolarizing factor; PGI_2 = prostaglandin I_2; ACE = angiotensin-converting enzyme; Ang = angiotensin; ET-1 = endothelin-1; cGMP = cyclic guanosine monophosphate; cAMP = cyclic adenosine monophosphate; 5-HT = 5-hydroxytriptamine.

eNOS expression in endothelial cells, likely because of the increased shear stress caused by the high cardiac output that accompanies sustained exercise.[48] In contrast, oxidized LDL, hypoxia and inflammatory cytokines such as TNF-α decrease eNOS expression.[10,49,50] The HMG-CoA reductase inhibitors increase eNOS levels by stabilizing the eNOS mRNA. This is thought to be an important component of the so-called pleiotropic effects of the statins, which may contribute to their therapeutic effects.

ENDOTHELIUM-DERIVED HYPERPOLARIZING FACTOR

Shortly after the identification of NO, it was suspected that the endothelium could release more than one relaxing factor, depending on the vessel size, the stimulus, and the species studied. Initial studies showed that some vasodilators produce hyperpolarization of the vascular smooth muscle membrane in an endothelium-dependent manner. It is now clear that this is due to the release of a hyperpolarizing factor from the endothelium that is almost certainly different from NO.[51] Its production is stimulated by many of the same stimuli that evoke the release of NO and depends on intracellular calcium. While there is some debate regarding the nature of this factor, increasing evidence suggests that it is a cytochrome P450 metabolite of arachidonic acid and perhaps other fatty acids.[52] When released from the endothelium, this epoxide opens calcium-activated potassium channels in the adjacent vascular smooth muscle, resulting in vasodilation.[53] Hydrogen peroxide has also been suggested to be a hyperpolarizing factor and has recently been shown to be responsible for flow-induced dilatation of human coronary arterioles.[54]

PROSTACYCLIN

Prostacyclin, or prostaglandin I_2 (PGI_2), a prostanoid derived from the action of cyclooxygenase on arachidonic acid, is released by the endothelium and relaxes vascular smooth muscle by increasing its intracellular content of cyclic AMP.[55] Prostacyclin is also platelet suppressant and antithrombotic and reduces the release of growth factors from endothelial cells and macrophages.[24] Among the agonists that stimulate prostacyclin synthesis are bradykinin (one of the most potent), substance P, platelet-derived growth factor and epidermal growth factor, and adenine nucleotides,[24] whereas aspirin has been shown to inhibit it transiently. Prostacyclin has been shown to compensate for the loss of NO in the eNOS knockout mouse.[56] Analogues of prostacyclin such as iloprost have proven useful in the treatment of pulmonary hypertension.[57]

ANGIOTENSIN-CONVERTING ENZYME

Endothelial cells, particularly those in the pulmonary vasculature, synthesize and express angiotensin-converting enzyme (ACE)[58] on their surface. ACE converts angiotensin I to the potent vasoconstrictor angiotensin II and degrades and inactivates bradykinin. Of note, vascular and cardiac cells contain almost all components of the renin/angiotensin system[59]; thus local production of angiotensin II can contribute importantly to vascular function. This local production of angiotensin II can explain why ACE inhibitors and angiotensin receptor antagonists are often effective even when the circulating levels of renin or angiotensin II are not elevated.

ENDOTHELINS

The endothelins are a family of closely related peptides made and secreted by many cells, including endothelial cells. There are three endothelins (ET-1, 2, and 3), all of which comprise 18 amino acid peptides. The endothelins are initially synthesized as preproendothelin, which undergoes preprocessing to big endothelin. Big endothelin is released and converted to active endothelin by the endothelin-converting enzyme. The vascular effects of endothelin are mediated by endothelin receptors, of which three subtypes have been identified (ET-A, B, and C). The receptors have differing specificity for the individual endothelin peptides, and activate different signaling pathways. In the vessel, the ET-A receptor is predominantly found on vascular smooth muscle, whereas the ET-B receptor resides on endothelial cells. Activation of the former stimulates potent vasoconstriction, whereas activation of the latter stimulates release of NO and thus favors vasodilation.[60]

The slow, intense, and sustained contraction caused by ET-1 appears to be the result of activation of the phosphoinositide/protein kinase C signaling pathway, as well as of opening voltage-dependent L-type calcium channels.[61] Importantly, even low, subthreshhold concentrations of ET-1 enhance vasoconstriction to a variety of other vasoconstrictor agents, including serotonin, angiotensin II, and α-adrenergic agonists, seemingly via activation of protein kinase C. This has been suggested to contribute to the *rebound phenomenon* that occurs after nitroglycerin has been administered for several days and is suddenly discontinued.[62]

ET-1 is also a potent growth factor for VSMCs[63] and a chemoattractant for monocytes.[64] Importantly, angiotensin II has been shown to stimulate the production of ET-1 by VSMCs in culture[65]; in vivo, some of the hypertensive effect of angiotensin II is mediated by endothelin.[66] There is substantial interest in the notion that ET-1 and angiotensin II may act in concert in conditions such as hypertension and heart failure, or that many of the effects of angiotensin II are mediated by ET-1.[67]

Endothelial Responses to Hemodynamic Influences

Many of the endothelial functions described above are modulated by the physical forces of stretch, strain, and shear stress imposed by the hemodynamics of the circulation. Flow-mediated, endothelium-dependent vasodilation has been described in many vascular beds,[68] and shear stress has been proposed to play a role in controlling endothelial cell proliferation.[69] Both stretch of the vessel wall (as observed in hypertension) and shear stress have been shown independently to affect endothelial cell morphology and/or function. Studies in cultured cells have shown that stretching endothelial cells leads to changes in cell shape,[70] intracellular signal generation with an increase in calcium and superoxide levels,[71] and proliferation.[69] Shear stress has numerous effects on endothelial cells. Initially, it was found that exposure of endothelial cell monolayers to elevated shear stresses in vitro caused them to align in the direction of flow. This reorientation was accompanied by changes in the cytoskeleton of the cells, including reorganization and alignment of the actin filaments and microtubules (Fig. 7-3). Similar mechanisms presumably also account for the orientation of endothelial cells parallel to the longitudinal axis in areas of laminar flow in the arterial system. The function of the endothelium is also altered by shear stress. Some of the cellular responses to shear stress include activation of K^+ currents; increased secretion of vasoactive and growth factors, including NO, endothelin, prostacyclin, and basic fibroblast growth factor (bFGF); enhanced tissue factor expression; elevation of LDL uptake; and increased tPA secretion.[72]

The importance of these observations lies in the variation in hemodynamic forces throughout the circulation. Areas of the vasculature exposed to low shear stress (branch points and curvatures) exhibit a predilection to the formation of atherosclerotic lesions.[73] True oscillations of flow have also been shown to occur in the carotid bulb, the proximal coronary arteries, and the distal aorta.[74] Studies in cul-

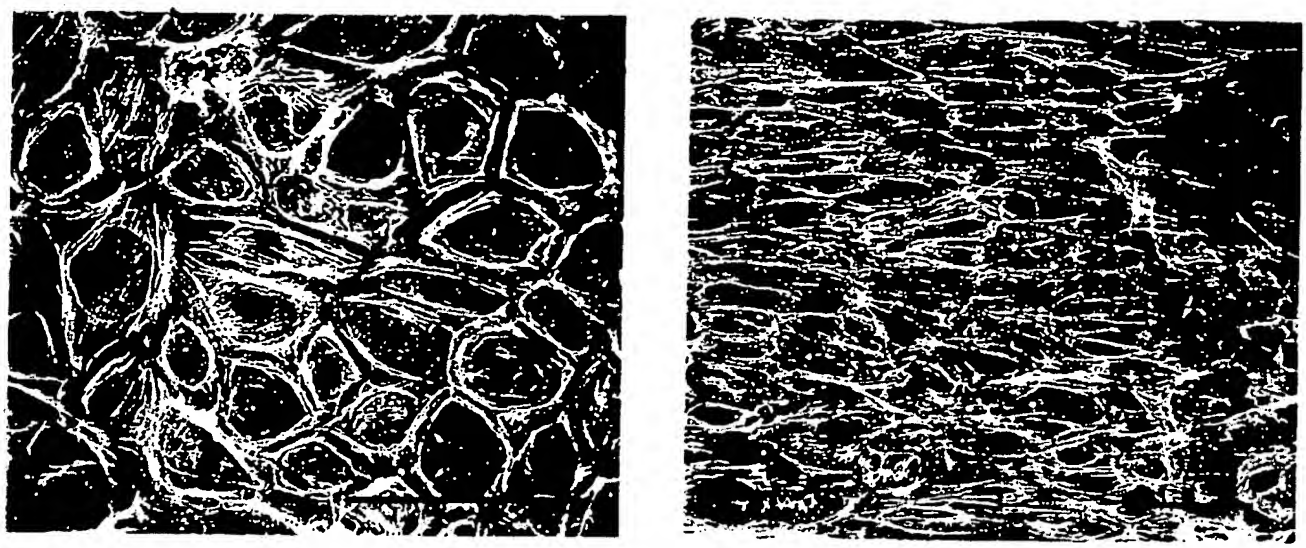


FIGURE 7-5 Effect of shear stress on endothelial cells. In bovine aortic endothelial cells grown in static conditions, F-actin filaments assume a random orientation as visualized by rhodamine-labeled phalloidin staining (left). Upon exposure to shear stress (30 dynes/cm^2, 24 h), these filaments align (right). Bars = 100 μm. (Courtesy of Lula Hilenski, Ph.D.)

tured endothelial cells have shown that oscillatory shear stress increases endothelial cell production of reactive oxygen species,[75] enhances adhesion molecule expression, and stimulates monocyte adhesion.[76]

The mechanisms by which the endothelial cell can sense and transduce mechanical signals have not been definitively determined. An attractive hypothesis deals with the concept that mechanical forces are transduced by integrin-mediated modifications of the endothelial cell cytoskeleton. Changes in the cytoskeleton, in turn, may have enormous effects on the biology of the endothelium. As an example, integrin activation leads to stimulation of kinases and phosphatases in focal adhesion complexes.[77] Changes in the actin cytoskeleton may affect RNA stability and translation.[78] In addition, flow-sensitive ion channels[79] and G proteins may be involved in mechanotransduction.[80] Furthermore, caveolae, which are flask-shaped membrane vesicular structures, are rich in signaling molecules such as G proteins and may be involved in signal generation in response to shear stress.[81]

PHYSIOLOGY OF THE VASCULAR SMOOTH MUSCLE CELL

The smooth muscle cell normally responds to hormonal stimulation with contraction or relaxation. In certain disease states, however, growth and/or hypertrophy and migration to the intima are the predominant responses. Some of the biochemical signals generated by these vasoactive agonists are similar for both types of responses, with the final physiologic response dictated by the phenotype and environment of the cell and the exact biochemical pathways activated.

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Some of the earliest signals generated within the cell following stimulation with calcium-mobilizing vasoactive agonists involve hydrolysis of a specific class of membrane lipids, the phosphoinositides, by phospholipase C.[82] This event leads to production of inositol trisphosphate (IP_3) and diacylglycerol (Fig. 7-4). IP_3 binds to a specific receptor on the sarcoplasmic reticulum, initiating release of Ca^{2+} from intracellular stores.[83] Ca^{2+}, in turn, activates a cascade of enzymes leading to contraction or growth (see below). Diacylglycerol is a potent activator of protein kinase C, a Ca^{2+}- and phospholipid-dependent enzyme that phosphorylates numerous cellular proteins and thereby enhances contraction at any given level of intracellular calcium.[84] Diacylglycerol can be further metabolized to phosphatidic acid or to glycerol, fatty acids, and, ultimately, eicosanoids and leukotrienes that may themselves modulate tone. Additionally vasoconstrictor agents cause an influx of extracellular Ca^{2+},[85] which serves to sustain and enhance vasoconstriction.

Contractions induced by various vasoactive hormones differ not only in magnitude and time course, but also differ between vessels. In general, there is an initial, rapid component of force generation and a more sustained phase of contraction. Some agonists, such as angiotensin II, induce only a transient constriction of many vessels, whereas others, including norepinephrine and vasopressin, nearly always cause a sustained contraction. The initial phase of force development has been shown to depend on the formation of actin-myosin crossbridges in response to acute elevations of intracellular calcium, whereas the sustained phase of contraction persists even after calcium levels return toward baseline.

A sliding-filament mechanism similar to that found in skeletal muscle is thought to regulate phasic contraction of smooth muscle. Tension development is regulated by the phosphorylation of the myosin light chain (Fig. 7-5) by an enzyme known as myosin light-chain kinase (MLCK). This protein associates with calmodulin, a calcium-binding protein required for activation of numerous cytoplasmic enzymes. Thus, when Ca^{2+} increases within the cell in response to hormonal stimulation, it binds to calmodulin, which, in turn, associates with MLCK, converting it from an inactive to an active form. MLCK then phosphorylates the myosin light chain, enabling actin activation of the Mg^{2+}-ATPase and ultimately resulting in crossbridge formation. When the intracellular Ca^{2+} concentration drops below about 100 n*M*, Ca^{2+} dissociates from calmodulin,

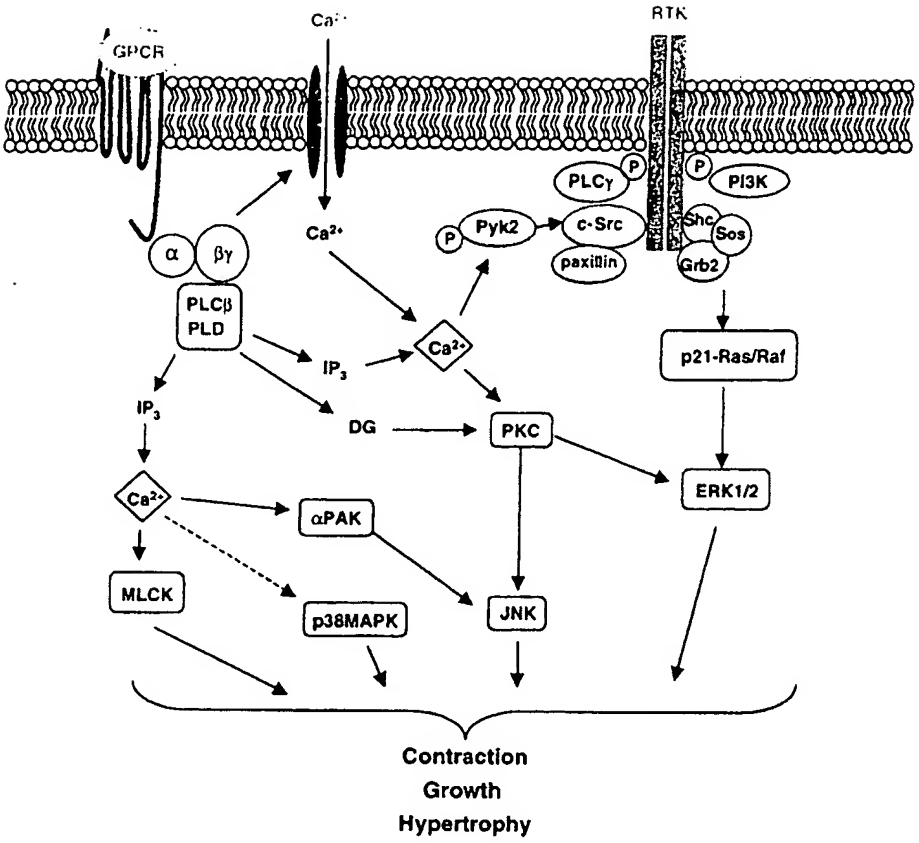


FIGURE 7-4 Signaling pathways in vascular smooth muscle. Vasoconstrictor agonists interact with specific G protein-coupled receptors (GPCRs) on vascular smooth muscle. These receptors are linked to a heterotrimeric G protein ($\alpha\beta\gamma$), which then couples to one or more phospholipase Cs (PLCs) or phospholipase D (PLD). PLC cleaves the inositol phospholipids to yield diacylglycerol (DG) and inositol phosphates, in particular, inositol trisphosphate (IP_3). IP_3 releases calcium from intracellular stores, and, along with DG, activates the Ca^{2+}- and phospholipid-dependent enzyme protein kinase C (PKC). Ca^{2+} activates numerous other kinases, including p21-activated kinase (α-PAK), Pyk2, and myosin light chain kinase (MLCK). PLD cleaves phosphatidylcholine to release phosphatidic acid, which is converted to DG. PKC is involved in activation of the mitogen-activated protein kinase (MAPK) cascade, including extracellular signal-regulated kinases (ERK1/2) and Jun kinase (JNK). Growth factors activate receptor tyrosine kinases (RTKs), Src, PLC-γ, and phosphatidylinositol 3-kinase (PI3K). RTKs also phosphorylate and form a signaling complex with paxillin and adapter proteins such as Shc, which binds Grb-2 and Sos and ultimately mediates the conversion of Ras to its active form. Ras phosphorylates Raf1, which in turn leads to activation of the MAP kinase cascade.

calmodulin detaches from MLCK, and MLCK becomes inactive. Myosin light chain phosphatase activity then predominates, myosin is dephosphorylated, and cross-bridge cycling ceases. During sustained contraction, however, the intracellular Ca^{2+} concentration is low, and energy consumption is reduced, suggesting the development of a latch-bridge, or a low cycling state.[86] Alternatively, the sensitivity of the contractile apparatus to Ca^{2+} may be increased, a response posited to be regulated by protein kinase C.[87] Recent evidence indicates that this latch state is also modulated by the actin-binding proteins caldesmon and calponin.[88] Caldesmon tonically inhibits contraction. Agonists such as phenylephrine stimulate extracellular regulated kinase (ERK1/2) mediated phosphorylation of caldesmon and enhance binding of calcium/calmodulin, removing its inhibitory effect and increasing tension.[89] Calponin has been suggested to directly inhibit the ATPase activity of myosin and to act as a signaling molecule that facilitates agonist-stimulated activation of protein kinase C.[90,91]

Recently it has become apparent that the small GTPase Rho, initially described as a modulator of the actin cytoskeleton, plays an important role in vascular smooth muscle contraction. In its active GTP-bound form, Rho activates Rho kinase, which inhibits myosin phosphatase type 1.[92] This inhibition in turn sustains MLC phosphorylation and sensitizes the contractile apparatus to calcium.[93] Rho kinase has become a target of therapeutic interventions. Its activity seems to be increased in hypertension,[94] and inhibitors of Rho kinase such as fasudil have been shown to lower vascular resistance in hypertension.[95]

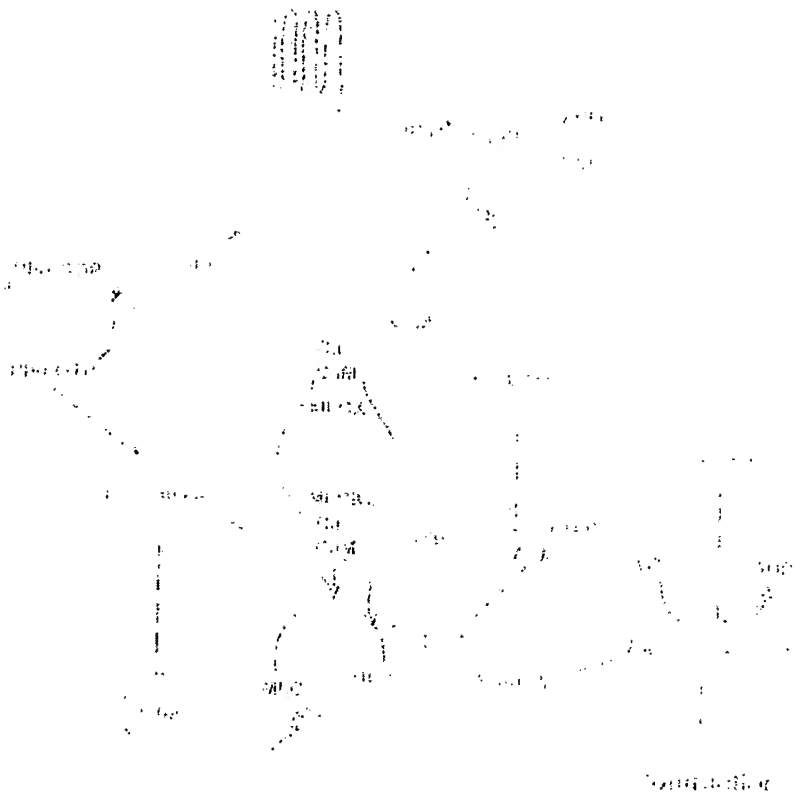
Factors Modulating Vascular Smooth Muscle Growth and Hypertrophy

Normally, vascular smooth muscle cells are relatively refractory to growth stimuli and exist in a quiescent, differentiated state. The healthy endothelium is critically important in maintaining this phenotype. Products of the endothelium such as nitric oxide,[96] prostacyclin,[96] heparan sulfates,[97] and transforming growth factor beta (TGF-β)[98] directly inhibit vascular smooth muscle growth. The endothelium is also an effective barrier limiting access of bloodborne growth factors to vascular smooth muscle. For example, the antithrombotic properties of the endothelium prevent access of promitogenic factors such as platelet-derived growth factor (PDGF) and thrombin to the underlying smooth muscle. Endothelial disruption allows initiation of a mitogenic smooth muscle response and regrowth of normal endothelium inhibits further proliferation.[99] In addition to these effects of the normal endothelium, the healthy vascular matrix minimizes vascular smooth muscle proliferation. It is impossible for cells to hypertrophy or proliferate without initial degradation of the matrix.

Under pathophysiologic conditions, the vascular milieu begins to favor vascular smooth muscle growth. Matrix metalloproteinases released by activated cells intrinsic to the vessel as well as invading inflammatory cells degrade the matrix to allow smooth muscle cell migration, proliferation, and hypertrophy. In addition, endothelial cells have the capacity to secrete several promitogenic agents. The best-studied of these factors is PDGF, so named because it was originally isolated from platelets. PDGF is a dimer, composed of two distinct peptide chains (designated A and B chains), and can be produced as an AB heterodimer or as an AA or BB homodimer. Endothelial cells contain the mRNA for both peptides.[100] Release of PDGF from the endothelium is regulated by growth factors, including TGF-β, fibroblast growth factor (FGF), and tumor necrosis factor

(TNF); circulating factors; and locally produced factors such as thrombin.[100] A second growth factor made and secreted by endothelial cells is insulin-like growth factor 1 (IGF-1),[13] which is a progression factor that facilitates movement of cells through the cell cycle and enhances the mitogenic effect of PDGF on smooth muscle.[101] IGF-1 production by endothelium is regulated by PDGF and plays a major role in vascular hypertrophy and hyperplasia.[102]

Other endothelial factors that affect smooth muscle proliferation include interleukin 1 (IL-1), FGF, and endothelin. IL-1 is an inflammatory cytokine that has numerous vascular effects in addition to mitogenesis, including the stimulation of procoagulant activity,[103] induction of leukocyte adhesiveness (see below), and inhibition of contraction.[104] Basic FGF has been detected in endothelial cells,[17] and acts as a potent smooth muscle mitogen, particularly after denuding injury.[105] It is stored in the subendothelial matrix and may be released by heparin and proteinases,[106] suggesting that the matrix may serve as a store for rapidly mobilizing this growth factor. FGF released from VSMCs may be particularly important in the growth response induced by injury to the arterial wall. Finally, endothelin-1, through its action on the ET-A receptor, induces smooth muscle cell growth by stimulating increases in intracellular calcium, activating protein kinase C and increasing intracellular production of reactive oxygen species. Diverse stimuli such as elevated insulin,[107] oscillatory shear stress and pressure,[108] and angiotensin II[109] potently induce endothelial production of ET-1.



Contraction cascade. Activation of smooth muscle by a vasoconstrictor hormone leads to a cascade of biochemical signals, ultimately resulting in phosphorylation of actomyosin, crossbridge formation, and force generation. The release of Ca^{2+} from intracellular stores is one of the major initiating events, since Ca^{2+} combines with calmodulin (CaM) to activate myosin light chain kinase (MLCK). This enzyme phosphorylates the myosin light chain (MLC), which is then able to interact with actin. In addition, activation of a guanine nucleotide exchange factor (GEF) for the low-molecular-weight G protein Rho leads to stimulation of Rho and Rho kinase, which inhibits myosin phosphatase (PP1M), thus enhancing myosin light chain phosphorylation (MLC-P). Caldesmon (CD), which normally inhibits actin-myosin interaction, becomes phosphorylated by extracellular signal regulated kinase (Erk1/2) and is released from this complex. Calponin acts by inhibiting myosin ATPase activity. $\alpha\beta\gamma$ = heterotrimeric G protein; PLC = phospholipase C; DG = diacylglycerol; PIP_2 = phosphatidylinositol 4,5-bisphosphate; IP_3 = inositol trisphosphate; Ca^{2+} = calcium; ATP = adenosine triphosphate; P = phosphate. (Courtesy of Bernard Lassègue, Ph.D.)

Regulation of Vascular Smooth Muscle Growth

Vascular smooth muscle cell growth occurs via two processes: hypertrophy and hyperplasia. In general, hypertrophy occurs in response to long-term stimulation with vasoconstrictor-type agents, whereas hyperplasia occurs in response to the classic growth factors. Hypertrophy is characterized by an increase in smooth muscle cell mass due to increased protein synthesis and has been shown to occur in response to angiotensin II[110] and thrombin[111] and in large vessels during hypertension. Hyperplasia is characterized by cell replication and is stimulated by growth factors such as PDGF and FGF following vascular injury.[105,112,113] The biochemical processes leading to hypertrophy and hyperplasia have been extensively investigated.

Classic growth factors, such as PDGF, activate many of the same signaling pathways as do vasoconstrictors: phosphoinositide hydrolysis, Ca^{2+} mobilization and influx, Na^+/H^+ exchange and intracellular alkalinization. Receptors for these growth factors are intrinsic tyrosine kinases, leading to the tyrosine phosphorylation of numerous proteins that are essential for growth. There is also increasing evidence that tyrosine phosphatases can counteract the mitogenic effects of growth factors by inhibiting tyrosine phosphorylation of specific substrates.[114]

A complex of substrates becomes associated with activated growth factor receptors and subsequently activates multiple signaling cascades leading to the final cellular response.[115] Upon stimulation, growth factor receptors dimerize and phosphorylate themselves on tyrosine residues. Some proteins, such as phospholipase C-γ, the tyrosine kinase c-Src, and phosphatidylinositol-3-kinase, bind directly to receptor tyrosine kinases, whereas others, including the tyrosine kinase Pyk-2 and the cytoskeletal protein paxillin, associate with the receptor via linker proteins such as Grb and Shc. Shc and Grb2 link these receptors to Ras, a ubiquitous GTPase that initiates a serine/threonine kinase cascade that includes mitogen-activated protein kinase (MAP kinase) and ultimately leads to growth. Recent evidence suggests that many of these proteins are also activated by seven-transmembrane-spanning G protein–coupled receptors,[116,117] an observation that may partially explain the growth-promoting properties of vasoconstrictor hormones like angiotensin II, thrombin and ET-1.

Components of the Extracellular Matrix

Proteoglycans	• Resistance to deformation • Arterial permeability, filtration, ion exchange • Transport and deposition of plasma elements • Regulation of cellular metabolism
Collagens (types I and III)	• Mechanical strength
Collagens (types IV, V and VI)	• Attachment of vascular cells to the matrix • Components of the basal lamina • Linking collagens to noncollagenous structures
Elastin	• Regulation of vascular elasticity
Fibronectin	• Cell-cell adhesion • Cell-substrate adhesion • Cell motility
Laminin	• Specific binding of collagen, heparin • Attachment of endothelial cells to type IV collagen

The extracellular matrix is a major component of the vessel wall. It is the medium through which nutrients are transported, a repository for products secreted by the cells of the vascular wall, the site of accumulation of cell debris, and a substrate for migration and proliferation of endothelial cells, monocytes, and vascular smooth muscle cells. The matrix consists of several proteins that have distinct functions in maintaining the integrity of the wall (Table 7-1).

Extracellular matrix degradation and reformation is an extremely important biological process with profound clinical implications. It is impossible for vascular cells to hypertrophy, proliferate, or migrate without an initial degradation of the matrix. One of the earliest events in angiogenesis is the degradation of the extracellular matrix to enable tube (capillary) formation. Vascular cells—including endothelial cells, VSMCs, resident macrophages, and fibroblasts—may secrete matrix metalloproteinases (MMPs), enzymes that selectively digest the individual components of the matrix. In addition, these cells elaborate tissue inhibitors of metalloproteinases (TIMPs).[8]

MMPs belong to three main groups: the type IV collagenases (also called *gelatinases*), the stromelysins, and interstitial collagenase. The characteristics of these proteins are described in Table 7-2.

In the past several years, it has become evident that reactive oxygen species play a crucial role in modulation of growth-related signaling pathways. Growth-promoting agonists stimulate the vascular NAD(P)H oxidases to produce superoxide and hydrogen peroxide, which may serve as progenitors to numerous other reactive oxygen species.[118,119] Of these, hydrogen peroxide seems to be particularly important in the growth process. Production of small concentrations of endogenous hydrogen peroxide activates specific mitogenic signaling pathways, such as p38 mitogen-activated protein kinase and Akt/protein kinase B, and promotes entry into the cell cycle.[120–122]

Matrix Metalloproteinases and Inhibitors

Interstitial collagenase	MMP-1	~45	VSMC, EC, microvascular EC	Inducible by PDGF, PMA, IL-1, VEGF
Type IV collagenase	MMP-9 gelatinase B type V gelatinase	92	VSMC EC	Inducible by IL-1α, PMA Inhibited by retinoic acid
	MMP-2 gelatinase A type IV gelatinase	72	VSMC wounded EC microvascular EC	Constitutive ↑ by TNF-α, IL-1α (VSMC) ↑↓ by retinoic acid (EC)
Stromelysin	MMP-3	50	VSMC EC microvascular EC	Inducible by IL-1 (VSMC); TNF-α, PMA (EC)
Matrilysin	MMP-7	...	VSMC, macrophage	Hypercholesterolemia
Membrane-type metalloproteinase	MT-MMP	..	VSMC, macrophage, EC	
TIMP-1	Inhibits MMPs	30	VSMC EC microvascular EC	Constitutive
TIMP-2	Inhibits MMP-2	~20	VSMC EC microvascular EC	Constitutive ↑ by retinoic acid (EC)

[a]The molecular weight of MMP-1 and MMP-3 depends on the species.

ABBREVIATIONS: EC, endothelial cell; IL, interleukin; MMP, matrix metalloproteinase; MT-MMP, membrane type MMP; PDGF, platelet-derived growth factor; PMA, phorbol 12,13-myristate acetate; TIMP, tissue inhibitor of metalloproteinase; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; VSMC, vascular smooth muscle cell.

MMPs are produced as inactive zymogens that can be activated by plasmin.[9] The activity of MMPs is also regulated by cytokines at transcriptional and posttranslational levels as well as by the relative levels of TIMPs. MMP-2 is usually found complexed with TIMP-2, its specific inhibitor.

In venous or microvascular endothelial cells, MMP-1 (interstitial collagenase), MMP-2 (72-kDa gelatinase), and TIMPs-1 and 2 are constitutively expressed. Although MMP-3 is only weakly expressed, it can be induced synergistically by incubation of the cells with the cytokine TNF-α and with phorbol ester tumor promoters.[8] This treatment also induces MMP-9 expression. Since MMP-2 and TIMP-2 are unaffected by TNF-α, cytokine activation of endothelial cells can change the complement of metalloproteinases produced. In VSMCs, MMP-2 is constitutively expressed, whereas MMP-1, MMP-9 (92-kDa gelatinase), and MMP-3 (stromelysin) are induced by cytokines such as interleukin 1 and TNF-α.[9] Cytokines can also activate MMP-2 zymogen.[123] Thus, cytokine stimulation increases the range of active metalloproteinases secreted by smooth muscle cells to encompass proteases capable of degrading all the major matrix components. In contrast, although TIMP-1 and TIMP-2 are constitutively expressed by vascular smooth muscle, their expression is unaffected by cytokines.[9] The net effect of cytokines on the vascular wall may be to tip the balance between the production of MMPs and TIMPs in favor of extracellular matrix degradation and remodeling.

Of particular importance, several reactive oxygen species have been shown to stimulate both activation and expression of MMPs, in particular MMP-9.[124,125] This is likely to be very important in diseases like atherosclerosis and hypertension, where vascular oxidant stress is increased. Activated macrophages accumulate at shoulder regions of the atherosclerotic plaque and secrete both MMPs and reactive oxygen species,[125,126] contributing to plaque rupture in this region.

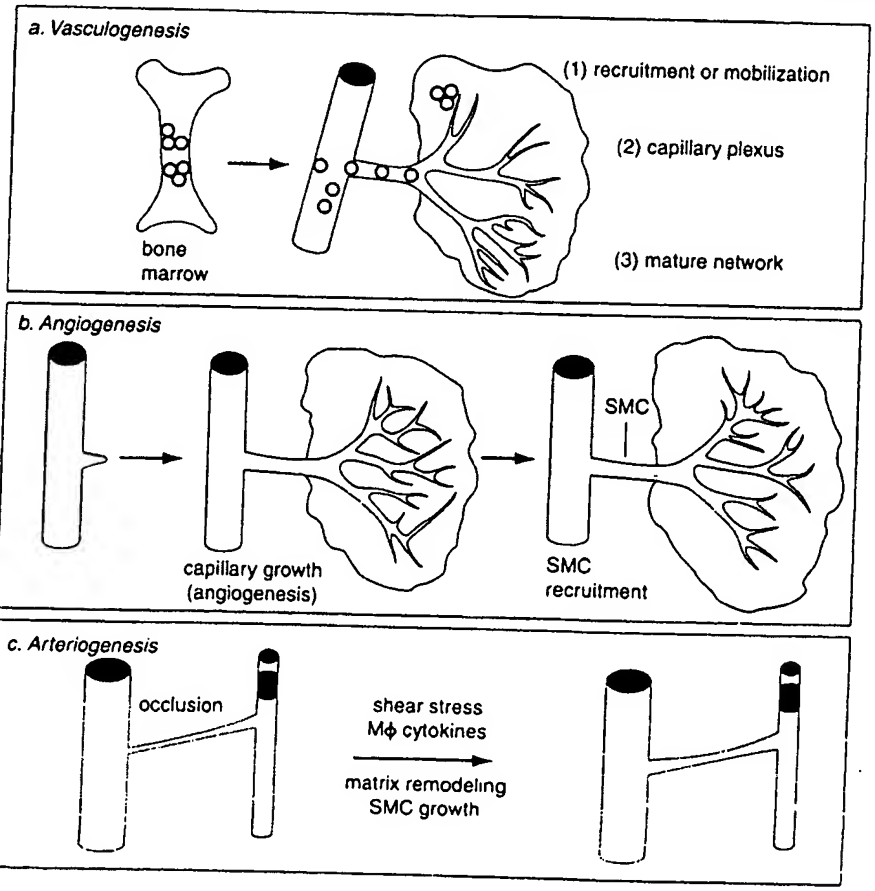
There has been a great deal of interest recently in the pivotal role of MMPs and TIMPs in vascular remodeling and atherogenesis.[127] A considerable body of evidence now exists that suggests vascular remodeling, as reflected in outcomes as disparate as a stenotic atherosclerotic lesion or an atherosclerotic aneurysm, is caused by varying combinations of major drivers of MMP-mediated remodeling–oxidative stress, inflammation, injury, and hemodynamics. The relative strengths of these different modulators of MMPs and their inhibitors appear to be major determinants of clinical outcomes, including atherosclerotic plaque regression, progression, or rupture, aneurysm formation, and adaptive or maladaptive angiogenic responses.[127–130]

[illegible]

The development of the vascular support for organogenesis during embryogenesis (vasculogenesis) is coming to be well understood at the most basic levels (see also Chap. 8).[131] A great interest in this process has developed among cardiovascular scientists and clinical investigators recently as it has become apparent that the normal vasculature is not necessarily, as previously assumed, a totally stable, static system, with minimal turnover of the endothelial and vascular smooth muscle cells. Although vascular remodeling in, for example, atherosclerosis was widely appreciated, it was thought previously that any new arterial formation, such as collaterals in ischemic coronary disease, arose only from cells from the existing vascular structures (angiogenesis and arteriogenesis), and the processes involved were poorly understood. These general perceptions have changed radically and rapidly with dramatic increases in understanding of the cellular and molecular mechanisms involved in blood vessel formation (Fig. 7-6). Another major impetus for a paradigm shift has been the appreciation that endothelial and vascular smooth muscle progenitor cells, which are likely derived from the bone marrow, contribute to and perhaps guide not only new blood vessel development in adults but also probably contribute to continuous renewal of existing vasculature.[132]

Vascular Development

The angioblast/hemangioblast is a very early endothelial cell progenitor and is also the progenitor cell for hematopoietic cells and skeletal muscle. Many of the genes and gene products (proteins) that are involved in vasculogenesis have been defined using mouse gene



Pathologic vascular growth in the adult may occur via vasculogenesis (endothelial progenitor cell (EPC) mobilization), angiogenesis (sprouting), or arteriogenesis (collateral growth). Mϕ macrophages; SMC smooth muscle cells (From Luttun et al.[132] With permission.)

Endothelial cell differentiation Proliferation Tube Formation	Vascular Branching and Remodeling	Pericyte Recruitment	Maintenance of Mature Vessel	Ischemia-Induced Angiogenesis
VEGF FGF2 TGF-β1	VEGF FGF ANG1	VEGF ANG1 PDGF-BB	ANG1	VEGF FGF2 ANG2 PDGF-BB PlGF

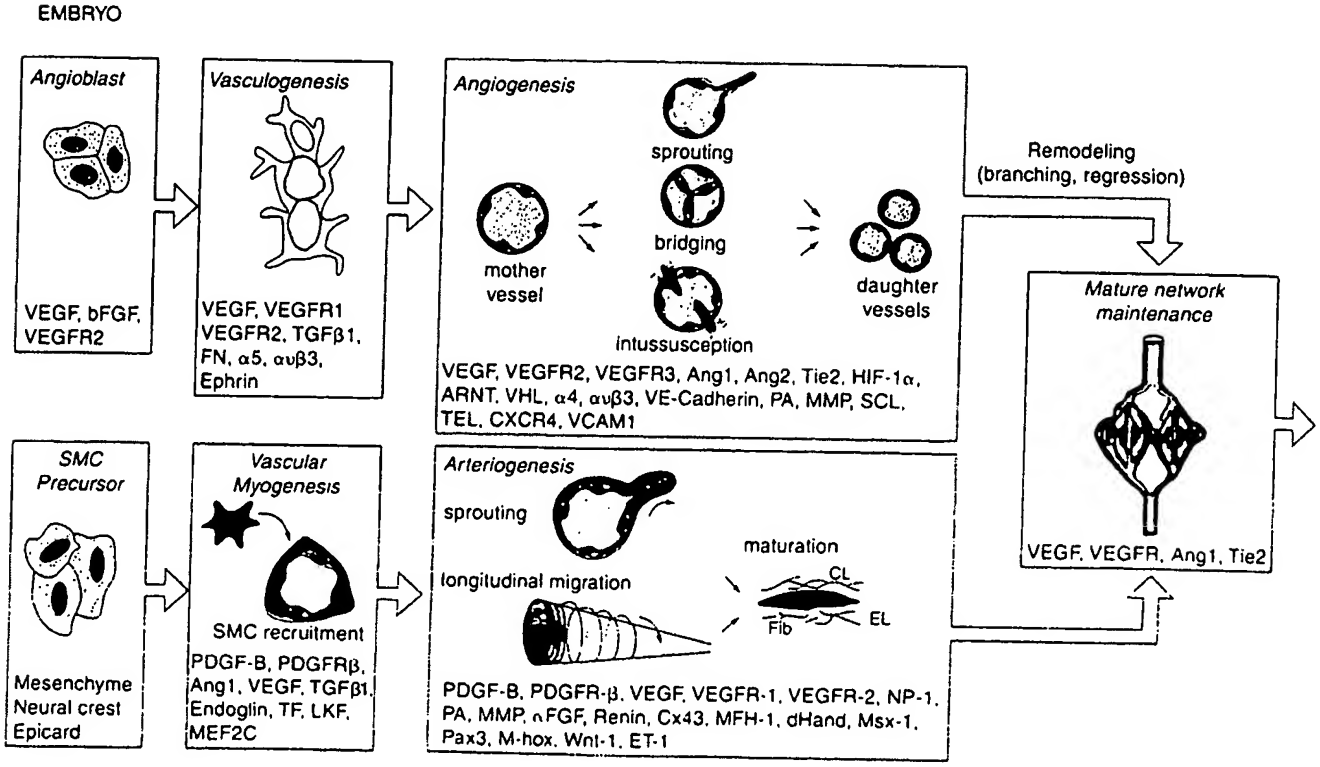
A simplified scheme of vasculogenesis and angiogenesis. Examples of angiogenic factors that are critical at each step are shown. VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; TGF-β1, transforming growth factor-β1; ANG1, angiopoietin-1; PDGF-BB, platelet-derived growth factor-BB (B - B chain); PlGF, placenta-derived growth factor. (From Semenza.[133] With permission.)

knockout models.[133] Of the large number of genes involved, a smaller number are particularly interesting because of the magnitude of the effects on vasculogenesis of knocking out the gene or of the potential of the encoded protein to promote angiogenesis in disease states. The vascular endothelial growth factor (VEGF) family is among the most important and VEGF is required for initial endothelial cell differentiation and proliferation.[134,135] VEGF binds to its cognate tyrosine kinase receptor VEGF receptor-2 (VEGFR-2), which is also known as flk1 or kdr.[136] Other homologues of VEGF include VEGF-B and placental growth factor (PlGF), and all three growth factors bind to VEGFR-1 or flt-1. The PlGF-flt-1 interaction is likely to be very important in pathological angiogenesis in the adult as discussed subsequently. Other growth factors, the angiopoietins (1 and 2), are important in the development and maintenance of mature blood vessels including the recruitment of pericytes, which are essential to the maturation process. An additional family of molecules that are important in blood vessel development into a network of arteries and veins are the ephrins, which are involved in cell-cell recognition.[134,135] Other growth factors that play a role in vascular development include platelet-derived growth factor BB (PDGF-BB), fibroblast growth factor (FGF), FGF2, and TGF-β1. The process of embryonic vasculogenesis, angiogenesis, and arteriogenesis is depicted sequentially with the involved growth factors in Fig. 7-7 and graphically in Fig. 7-8.

Angiogenesis and Arteriogenesis in the Adult

GROWTH FACTORS

In animal models of ischemic heart disease, VEGF expression is stimulated after coronary artery occlusion and is associated with the development of collateral formation.[137,138] VEGF expression is regulated, at least in part, by hypoxia-inducible factor 1 (HIF-1), which is a transcription factor that acts as a molecular switch for angiogenesis genes.[139] HIF-1 expression is upregulated by conditions of



Endothelial precursors (angioblasts) in the embryo assemble in a primitive network (vasculogenesis) that expands and remodels (angiogenesis). Smooth muscle cells cover endothelial cells during vascular myogenesis and stabilize vessels during arteriogenesis. CL - collagen; EL - elastin, Fib - fibrillin. (From Carmeliet.[255] With permission.)

Clinical Trials of Therapeutic Angiogenesis

FGF-1	Protein
FGF-2	Protein
FGF-4	Adenovirus
$VEGF_{121}$	Adenovirus
$VEGF_{165}$	Plasmid
$VEGF_{165}$	Protein
VEGF-2	Plasmid
Transcription factor	**Mode of administration**
HIF-1α/VP-16 fusion protein	Adenovirus

ABBREVIATIONS: FGF = fibroblast growth factor; VEGF = vascular endothelial growth factor; HIF = hypoxia-inducible factor
SOURCE: From Semenza.[133] With permission.

hypoxia and ischemia. The response of this system, which is considerably more complicated than outlined here, is obviously very variable, as reflected in clinical experience in which different patients may have robust or no obvious collateralization with apparently similar degrees of coronary artery obstruction. This heterogeneity of individual responses appears to be dependent in part upon age as determined in animal experiments.[140-142] In addition to age-related factors, evidence suggests that individual genetic or environmental factors, predictably, may determine the responsiveness of the systems controlling collateralization in humans.[133,143]

The logic that inadequate collateralization clinically could be compensated for by the administration of either angiogenic growth factors or the genes encoding them has led to a number of clinical trials (Table 7-3).[144,145] Clinical efficacy has not been clearly demonstrated as yet. The approaches in these trials of using a single angiogenic factor such as VEGF or FGF may be conceptually flawed. As noted earlier, the generation of a mature blood vessel requires mutiple factors in addition to VEGF or FGF. The potential problem is perhaps illustrated by the fact that angiopoietin 1, which interacts with the receptor Tie-2, is essential to stabilize mature blood vessels, which are, among other things, very leaky in its absence.[146] In animal models, VEGF infusion not only stimulates formation of leaky blood vessels but can stimulate formation of hemangioma-like structures.[132] On the other hand, it has recently been shown in animal models that infusion of PlGF is more robust than VEGF in revascularization of ischemic tissues and in the induction of formation of stable, mature (imbued with vascular smooth cells and pericytes) blood vessels in this setting.[136] The effect of PlGF was attributed, in part, to its stimulation of flt-1 and to mobilization of bone marrow–derived inflammatory cells to ischemic regions. PlGF thus may be a more attractive candidate than VEGF (or FGF) for therapeutic angiogenesis using growth factor delivery to ischemic tissues as the principal strategy.

BONE MARROW–DERIVED PROGENITOR CELLS

As noted, neovascularization in the adult previously was thought to result exclusively from angiogenesis.[132] Vascular progenitor cells (hemangioblasts) were identified earlier and were characterized as endothelial progenitor cells (EPCs) in studies of embryonic vasculogenesis.[147] Several recent studies, moreover, have described EPCs circulating in the peripheral blood postnatally that incorporated into the neovasculature and were associated with tumors, ischemic myocardium and hindlimbs, cutaneous wounds, and injured corneas.[148-151] Utilization of EPCs, whether derived from the bone marrow directly (or indirectly by stimulating the release out of the marrow with cytokines or growth factors) or by expanding the relatively small population of circulating EPCs in vitro, has enormous biological significance and therapeutic potential. The burgeoning literature on the subject has recently been reviewed in detail generally[132] and specifically with respect to clinical applications.[152,153]

[illegible]

Endothelial cells actively participate in the development of inflammatory reactions. The recruitment of leukocytes to sites of inflammation is initiated by endothelial secretion of chemotactic molecules and enhanced expression of adhesion molecules that interact with surface proteins on leukocytes.[154] Cytokines and arachidonic acid metabolites of the leukotriene pathway derived from cells of the vessel wall, the infiltrating macrophages and T-lymphocytes stimulate endothelial secretion of many of these molecules.[154] An important new class of molecules that mediate the vascular inflammatory response comprises the chemokines, of which more than 50 have been identified.[155] Two classes of the chemokines exist, known as the CXC and CC, based on differences in the position of the first two cysteines in their amino acid sequence. These interact with at least 20 G-protein receptors, classified as CXCR and CCR, according to their corresponding ligand.

It has been suggested that the sequential accumulation of different leukocyte classes at sites of inflammation can be explained by the differential induction of these endothelial cell adhesion molecules.[156] An early step in the inflammatory response is capture of leukocytes from the flowing blood, mediated by the L and P selectins.[157,158] A second step is leukocyte rolling, which is mediated by interaction with E and P selectins.[157,158] Ultimate adhesion of leukocytes is dependent on interactions with β2-integrins PECAM and ICAM-1.[157,158] At the site of atherosclerotic lesions, recruitment of leukocytes is markedly enhanced by the surface expression of VCAM-1 and interactions between the monocyte chemotactic protein MCP-1 with its monocyte receptor CCR2.[159,160] IL-8 and its receptor CXCR2, as well as fractalkine and its receptor CX_3CL1, also contribute to this response.[161,162] The list of proinflammatory molecules contributing to these endothelial/leukocyte interactions continues to grow, and it is likely that the complete inflammatory response depends on the concerted action of many such molecular mediators. This is supported by the observation that genetic knockout of either the IL-8 receptor, MCP-1, or CX3CR1 each leads to a >50 percent reduction in lesion formation in atherosclerotic mice.[161,162]

[illegible]

In general, the normal endothelium is in an inhibitory mode—inhibiting contraction, thrombosis, white cell adhesion, and vascular smooth muscle growth (Figs. 7-2 and 7-9). A common feature of many different vascular diseases is that these functions of the endothelium are either lost or disrupted, a phenomenon often referred to as *endothelial dysfunction*. Implicit in this term is the recognition that the fundamental or normal functions of the endothelium are not fixed but are mutable. Thus, the endothelium in a given area may lose its vasodilator predominance, become prothrombotic or less thrombolytic, begin to support leukocyte adherence (which may be a normal response in the inflammatory process), or stimulate rather than inhibit smooth muscle migration and proliferation.

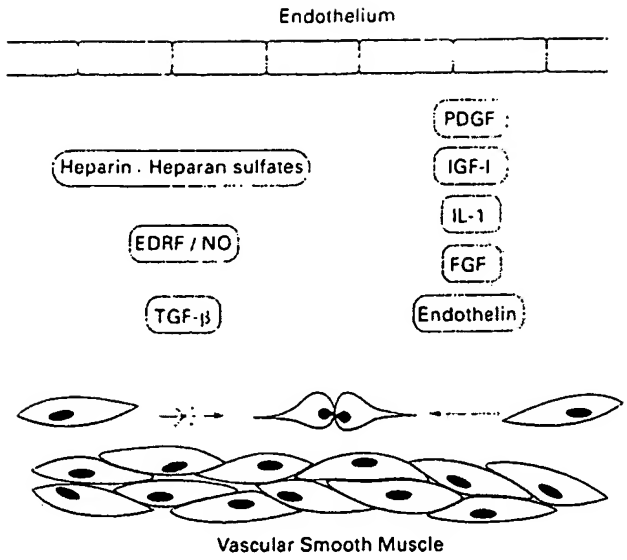


Fig. 7-9 Endothelial control of vascular growth. As with vasoactive substances, endothelial cells make and secrete both growth-promoting (*white boxes*) and growth-inhibitory (*shaded boxes*) compounds. Under normal conditions, the net effect of the endothelium is growth-inhibitory. EDRF = endothelial-derived relaxing factor; NO = nitric oxide; TGF-β = transforming growth factor beta; PDGF = platelet-derived growth factor; IGF-I = insulin-like growth factor-I; IL-1 = interleukin-1; FGF = fibroblast growth factor. (Courtesy of Bernard Lassègue, Ph.D.)

Oxidative Stress and Vascular Disease

In the past several years, it has become clear that vascular cells—including endothelial, vascular smooth muscle, and adventitial cells—can produce reactive oxygen species (ROS).[163] These include the superoxide anion, hydrogen peroxide, NO, and peroxynitrite. In numerous pathophysiologic conditions, the production of ROS in the vascular wall is increased, resulting in a situation commonly referred to as *oxidant*, or *oxidative, stress*. Several enzyme systems have been implicated in production of ROS.

Recent studies suggest that NAD(P)H oxidases are major sources of ROS in endothelial and vascular smooth muscle cells.[163] These are multisubunit enzymes that have partial similarity to the neutrophil respiratory burst oxidase. In phagocytic cells, two membrane components, $p22^{phox}$ and $gp91^{phox}$, comprise the cytochrome b558, which is regulated by cytoplasmic subunits, including $p47^{phox}$, $p67^{phox}$, and the small G protein Rac.[164] On a molecular level, the vascular oxidases share limited homology with the neutrophil respiratory burst oxidase. Many of the neutrophil components including $p22^{phox}$, $p47^{phox}$, and the small GTPase Rac are present in vascular cells. During the past few years, a family of proteins termed the Nox proteins with homology to $gp91^{phox}$, the neutrophil oxidase catalytic subunit, has been shown to play a critical role in function of both the smooth muscle and endothelial cell NAD(P)H oxidases.[165–167] Endothelial cells contain Nox1, Nox2, and Nox4, while vascular smooth muscle cells express Nox1, Nox4, and Nox5.[167,168] The adventitia also contains fibroblasts and macrophages that express multiple oxidase subunits.[169]

Importantly, the NAD(P)H oxidases are activated by several pathophysiologic stimuli, including angiotensin II, mechanical stretch, cytokines, and thrombin.[71,119,170,171] Recent studies have shown that the low-molecular-weight G protein Rac1 is a central regulator of oxidase activity.[172] Rac1 geranylation can be inhibited by the HMG- CoA reductase inhibitors,[173] suggesting one mechanism whereby these agents can have vasculoprotective effects.

A second source of ROS is eNOS. As discussed previously, in the absence of tetrahydrobiopterin or L-arginine, this enzyme becomes "uncoupled," so that it produces hydrogen peroxide and superoxide rather than NO.[41,174] Importantly, this uncoupling process seems to occur in several common disease states, including hypercholesterolemia,[175] hypertension,[42] and diabetes,[176] although the mechanisms responsible for this process are poorly understood.

An important source of radicals in the vasculature is the lipoxygenases, in particular 12,15-lipoxygenase.[177] These do not form superoxide but react directly with unsaturated fatty acids (e.g., linoleic and arachidonic acids) to form a lipid radical (L·), which in turn can react with molecular oxygen to produce alkoxy-radicals (LO·), and lipid peroxy radicals (LOO·). These lipid radicals are biologically very active and can stimulate gene expression, consume NO, oxidize NADH, and serve as a source of other radicals.[178]

Other sources of ROS in vascular cells are xanthine oxidase, cytochrome P450, cyclooxygenase, and mitochondrial electron transport.[178] There is now substantial interest in the role of these various sources of ROS and how they contribute to vascular oxidant stress.

In the next several paragraphs, we consider how endothelial dysfunction and vascular smooth muscle abnormalities contribute to several vascular diseases. A recurring theme in these conditions is that ROS play a central role. For example, superoxide rapidly reacts with NO, forming the strong oxidant peroxynitrite. The latter can oxidize lipids, damage lipid membranes, deplete cellular thiols, and alter function of several enzymes.[179] This inactivation of NO alters vasomotion and can predispose one to or even cause hypertension.[180] Other ROS such as the hydroxyl radical and lipid radicals can react with NO. Recently it has been recognized that peroxidases, upon reaction with H_2O_2, can consume NO. A substantial component of VSMC hypertrophy caused by angiotensin II is mediated by hydrogen peroxide.[181] ROS also contribute to vascular inflammation by stimulating expression of adhesion molecules in endothelial cells.[182] These issues are discussed in the context of several vascular diseases.

Atherosclerosis

Atherosclerosis is the prototypical disease characterized by endothelial dysfunction, which may explain many of its cardinal features. Thus, mononuclear and lymphocytic infiltration, vascular smooth muscle hypercontractility, modification of low-density liporotein (LDL), smooth muscle cell growth, and intimal migration are likely related to abnormalities of the endothelium induced by hyperlipidemia, hypertension, smoking, and unknown hereditary factors. The pathogenesis of atherosclerosis viewed as a disease of endothelial dysfunction is depicted in Fig. 7-10. (For a more detailed discussion, see Chap. 44.)

Clinically, endothelial dysfunction in atherosclerosis has primarily been defined by impairment of endothelium-dependent relaxation.[183,184] Coronary endothelium-dependent vasodilator function is impaired in patients with risk factors such as hypercholesterolemia and prior to angiographically demonstrable coronary disease.[185] As previously discussed, increased inactivation of NO by the superoxide anion is likely one cause of this abnormality.[186,187] Other causes may include "uncoupling" of the eNOS enzyme, altered calcium signaling of eNOS, and diminished expression of the eNOS enzyme, which clearly occurs late in the atherosclerotic process.[188] Of note, LDL and cytokines have been shown to downregulate eNOS by destabiliz-

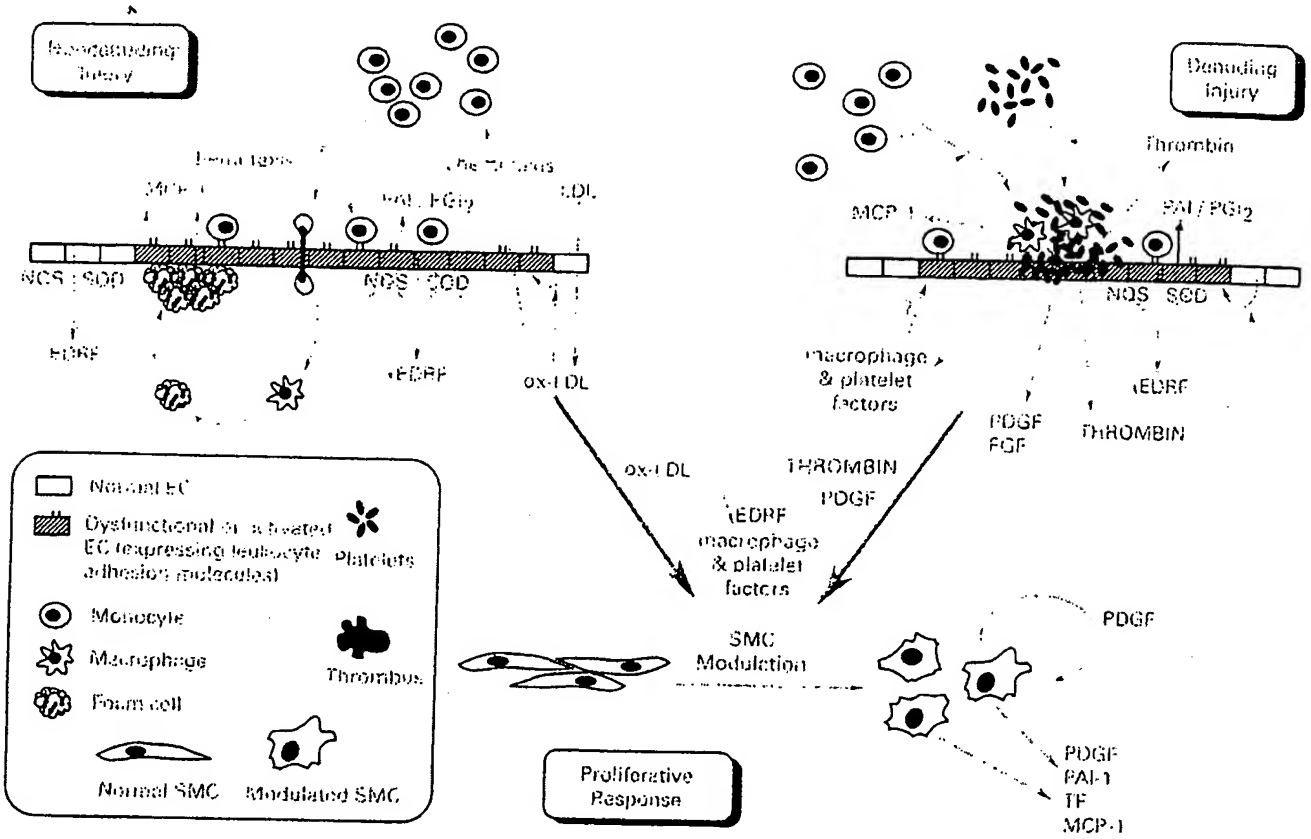


FIGURE 7-10 Theoretical initiating events in vascular lesion formation. Nondenuding injury: Low-density lipoprotein (LDL) enters the subendothelial space where it is converted to oxidized LDL (ox-LDL), which induces monocyte chemoattraction and endothelial dysfunction. Dysfunctional endothelial cells (ECs) express cell adhesion molecules (ICAM, ELAM, and VCAM), leading to increased monocyte adhesion and movement into the vessel wall. Monocytes in the vessel wall differentiate into macrophages, take up lipids, and remain locally as foam cells, subsequently evolving into fatty streaks. The foam cells in the fatty streak and the overlying endothelium express monocyte chemotactic protein 1 (MCP-1), resulting in further enhanced monocyte chemoattraction and adhesion. Dysfunctional ECs may synthesize less nitric oxide synthase (NOS) or superoxide dismutase (SOD, an enzyme that metabolizes oxygen radicals, which have been shown to inactivate NO). This decreases endothelium-derived relaxing factor (EDRF) release/activity. The loss of EDRF together with the direct effects of ox-LDL, or growth factors secreted by the foam cells or endothelium, act on the quiescent contractile smooth muscle cells in the vessel wall, giving rise to the proliferative phenotype, with division and migration into the intima. Denuding injury: Loss of endothelium leads to platelet deposition, tissue factor–mediated activation of extrinsic coagulation to generate thrombin, cleavage of fibrinogen to fibrin, and the formation of thrombus. Thrombin gives rise to endothelial expression of adhesion molecules and consequent monocyte attachment, together with secretion of platelet granular constituents. Monocytes enter the thrombus and differentiate into phagocytic macrophages expressing tissue factor and MCP-1. This leads to further monocyte chemoattraction into the vessel wall. Smooth muscle cell proliferation is produced by (1) thrombin generation at the site of denuding injury, (2) platelet-derived growth factor (PDGF) or other growth factors released from platelets in the thrombus, (3) factors secreted by the macrophages ingesting the thrombus, and (4) the loss of EDRF activity caused by endothelial dysfunction. *Proliferative response*: Modulated smooth muscle cells (SMCs) proliferate and synthesize factors that promote plaque development. SMCs synthesize (1) PDGF and other growth factors that cause self-perpetuating autocrine or paracrine stimulation of SMC proliferation, (2) tissue factor (TF) and plasminogen activator inhibitor 1 (PAI-1) that act locally to produce thrombin or inhibit fibrinolysis of the fibrin network used to facilitate cell migration, and (3) MCP-1, which increases monocyte chemoattraction into the lesion, thereby leading to lesion development. (We thank Drs. Laurence Harker, Josiah Wilcox, and Bernard Lassègue for their creative and intellectual contributions to the development of this figure.)

ing the eNOS mRNA. This is prevented by HMG-CoA reductase inhibitors even without lowering of cholesterol. New evidence suggests that this process involves the lipid modification of the small GTPase Rho by the attachment of a geranylgeranyl and lipid moiety which facilitates its localization to the cell membrane, suggesting a new target for the HMG-CoA reductase inhibitors.[189]

A second manifestation of a dysfunctional endothelium that is apparent very early after initiation of cholesterol feeding in animals is the recruitment of monocytes and macrophages into the vessel wall.[190] This recruitment is likely the result of induction of VCAM-1 expression[191] as well as secretion of MCP-1.[192] The molecular linkage between hyperlipidemia and MCP-1/adhesion molecule expression is unknown, but may reflect in part the oxidative stress imposed by this change in milieu. Inflammatory cytokines are also important mediators of adhesion molecule expression.[193] and their production by the endothelium and inflammatory cells in the vessel wall may also contribute to adhesion molecule expression in both the early and the late stages of the disease. Inhibition of MCP-1 or its receptor attenuates the development of early atherosclerotic lesions in experimental animals.[159,194] In addition, deletion of the fractalkine receptor (CX3CR1) reduces macrophage recruitment into the vessel wall and diminishes lesion formation in ApoE-deficient mice.[195] Moreover, a

common polymorphism of the fractalkine receptor has been shown to have reduced adhesive and chemoattractant properties and is associated with a lower incidence of human atherosclerosis and acute coronary syndromes.[195]

The intimal proliferation observed in atherosclerotic lesion formation results from migration and hyperplasia of vascular smooth muscle cells and myofibroblasts[196] and accumulation of extracellular matrix.[197] Proliferation has been attributed to growth factors such as PDGF, FGF, and IGF-1. Since these growth factors can be produced by the endothelium in vitro, it is very likely that the dysfunctional endothelium in atherosclerosis also produces growth factors while shifting from a growth-inhibitory to a growth-promoting mode. Furthermore, there is evidence that products of oxidative metabolism may increase matrix metalloproteinase activation[124] and expression,[125] thus contributing to intimal lesion formation on multiple levels.

The recent advances in our understanding of vessel wall biology provide insight into the biological mechanisms responsible for the pathogenesis of atherosclerosis. A unifying concept of the disease has arisen that revolves around endothelial dysfunction mediated by changes in oxidative metabolism. Oxidative stress and oxidatively modified LDL thus assume central roles in atherogenesis (Fig. 7-10). As discussed previously, a major source of lipid oxidation is lipoxygenases. Knockout of 12,15 lipoxygenase or the NAD(P)H oxidase subunit p47^{phox}[198,199] reduces atherosclerosis in ApoE-deficient mice. These data indicate that 12- and 15-lipoxygenases and the NAD(P)H oxidases are almost certainly involved in the atherosclerotic process. The role of oxidized LDL and the relationship of the cell biology of atherosclerosis to coronary ischemic syndromes are discussed in Chap. 44.

Hypertension

Hypertension is characterized by dysfunction of both endothelium and vascular smooth muscle. In chronic hypertension, endothelium-dependent relaxations are impaired in both conduit and resistance arteries.[200–203] Relaxations to some platelet factors are also altered, but have been found to be augmented or diminished, depending on the hypertensive model studied.[204] Furthermore, the endothelium-dependent constrictor activity is increased in some models of hypertension.[204] These alterations in endothelial function would tend to increase the tone of hypertensive vessels. Recently, it has been shown that hypertension is associated with oxidation of tetrahydrobiopterin by NAD(P)H oxidases, a critical cofactor for NO synthase. This leads to eNOS uncoupling, reducing NO production and increasing endothelial superoxide production. Removing the oxidant stress by either knockout of p47^{phox} or by replacement of tetrahydrobiopterin lowered blood pressure in experimental animals.[42,205]

Hypertension is also characterized by an increase in vessel wall mass. In the aortas of spontaneously hypertensive and Goldblatt hypertensive rats, this increase can be attributed to an increase in the size of the existing smooth muscle cells.[206,207] Hypertrophy is accompanied by an increase in ploidy; that is, an increased DNA content per cell.[206,207] In contrast, resistance vessels from these same animals appear to increase their mass by hyperplasia of the smooth muscle cells.[208] The stimuli responsible for these changes in the hypertensive vascular wall are unknown. Vascular remodeling appears to have two stages: (1) an initial, reversible, intense vasoconstriction mediated by neural or endogenous signals, followed by (2) a remodeling of the vessel wall characterized by increased smooth muscle mass and narrowing of the vessel lumen. There is some evidence that this response is dependent on the presence of the endothelium.[209]

When the endothelium becomes dysfunctional, as in atherosclerosis, the underlying smooth muscle cells often become hyperreactive to certain vasoconstrictor stimuli, including serotonin and ergonovine.[210] Coronary spasm leading to myocardial infarction is one of the most clinically relevant problems arising from this phenomenon. Proposed mechanisms underlying this vasoconstrictor abnormality that can result in total occlusion include supersensitivity of the smooth muscle cells to constrictor stimuli and loss of endothelium-dependent relaxing mechanisms. The increased tendency toward thrombus formation in dysfunctional endothelium, due to a loss of the normal anticoagulant properties, also promotes the release of thrombus-related factors (serotonin, thromboxane A_2, ADP, thrombin, and PDGF) in the vicinity of the smooth muscle cells, which can promote vasoconstriction.[211]

Restenosis

Restenosis is the development of a neointima that occurs following angioplasty, often leading to reocclusion of the initial lesion. The response of the arterial wall to the injury induced by angioplasty (removal of the endothelium and stretching of the vessel wall) involves several distinct events. Removal of the endothelium not only alters the paracrine hormonal environment in which VSMCs exist, but it also exposes a thrombogenic surface to which platelets and other circulating factors can adhere, resulting in the formation of a thrombus. In addition, injury to the underlying smooth muscle may release factors such as FGF, which have mitogenic effects on the remaining smooth muscle cells. Finally, infiltration and subsequent activation of macrophages into the denuded vessel wall bring an additional set of hormonal influences to bear on the vascular smooth muscle. The pathophysiologic consequences of these complex events include migration and proliferation of smooth muscle cells into the intimal area, resulting in the formation of a neointima over a period of weeks to months.

models, including pigs, rabbits, rats, and baboons. In the rat carotid artery, the events following injury can be divided into three stages: initial (injury to 48 h), migratory (3 to 7 days), and proliferative (7 days to 3 to 4 weeks). During the initial response to injury, growth-related genes in the smooth muscle cells are induced, including c-fos, PDGF-A, PDGF-β receptor and MCP-1.[212,213] It also appears that deep injury to smooth muscle cells results in an outpouring of FGF, a potent smooth muscle mitogen.[214] This initial response does not appear to depend on platelet factors, but does appear to be directly related to the removal of the endothelium.[99] During the migratory phase, a large increase of thymidine incorporation in the vessel wall occurs, accompanied by further increases in the mRNA encoding IGF-1[215] and the PDGF-β receptor.[212] This phase of the response can be modulated by platelet factors and inhibited by the endothelium.[99] Finally, the proliferative phase is characterized by marked intimal thickening, with a decreased percentage of thymidine-labeled cells. Some of the increased area is due to deposition of extracellular matrix, and the majority of the proliferative activity occurs at the luminal surface of the vessel. This proliferative phase seems ultimately to be inhibited by regrowth of normal-functioning endothelium.

Thus, during the process of restenosis after angioplasty, both the loss of endothelium and the transformation of smooth muscle cells appear to contribute to neointimal formation. At least two lines of evidence implicate the endothelium as having a crucial role in the response of the vessel wall to injury. First, removal of the endothelium

allows initiation of the mitogenic response and, second, regrowth of normal endothelium inhibits further proliferation. Furthermore, gentle denudation with a nylon loop accompanied by rapid regeneration of endothelium results in significantly less neointimal proliferation.[216] In addition, proliferating smooth muscle cells have characteristics distinct from those of the differentiated smooth muscle cells in the medial layer. Their cytoskeleton is similar to that found in cultured cells. It seems likely, therefore, that two of the most important causes of restenosis are the loss of endothelium-derived growth-inhibitory factors and the transformation of smooth muscle cells into a phenotype able to respond to platelet- and endothelium-derived factors with proliferation.

ROS are thought not only to be centrally involved in the pathogenesis of atherosclerosis but very likely also to be major mediators of the proliferative, hypertrophic, and fibrotic responses that frequently occur in arteries after percutaneous transluminal coronary angioplasty (PTCA), resulting in renarrowing or restenosis of the lumen (see Chap. 55). Migration and growth of VSMCs into the intima contribute significantly to restenosis, and intracellular signaling pathways mediating growth, hypertrophy, and migration are stimulated by ROS.[217,218] As discussed previously, both proinflammatory pathways and matrix metalloproteinases, which facilitate vascular remodeling, involve redox-sensitive controlling mechanisms. The apparent broad role for oxidative signaling mechanisms in the vascular wall led to testing of the concept that antioxidants might inhibit restenosis. The production of superoxide is increased in vessels following balloon injury.[219,220] Several clinical studies have shown that the potent antioxidant probucol and a newer, soluble probucol derivative can prevent restenosis after either balloon angioplasty or coronary stent placement.[221–224]

FUTURE DIRECTIONS

Defining the molecular and cellular basis for dysfunction of the arterial wall in vascular diseases provides information critical to developing clinical strategies for patient management, as well as to finding new therapeutic targets. It is now clear that both endothelial function and smooth muscle function are compromised by a variety of risk factors for vascular disease, due in part to oxidative stress. Further research is required to determine at a more basic level the molecular events that link these risk factors to these diseases. The human genome has been fully sequenced, and via the use of bioinformatics it will be possible to identify genetic profiles that predispose people to the development of vascular pathologies. Clinical trials in the future will be targeted to these populations in new and powerful ways, and basic research will address the roles of these newly identified genes in vascular physiology and pathophysiology. In addition, the advent of stem cell technology opens new avenues for therapeutic treatment. Once researchers understand the mechanisms controlling stem cell targeting and differentiation, genetic and pharmacologic manipulation of these cells may be the treatment of the future.

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EXHIBIT B

The coronary artery bypass conduit: I. Intraoperative endothelial injury and its implication on graft patency

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Ann Thorac Surg 2001;72:2245-2252

The Coronary Artery Bypass Conduit: I. Intraoperative Endothelial Injury and Its Implication on Graft Patency

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Prevention of intraoperative injury to the vascular endothelium is of primary importance in maintaining viability and patency of the aorto-coronary saphenous vein graft. Surgical manipulation, ischemia, storage conditions, and distension before anastomosis can abnormally alter the antithrombogenic property of the endothelium leading to vasospasms, thrombogenesis, occlusive intimal hyperplasia, and stenosis. Endothelial injury can also form an initiation site for the formation of later-stage atheromas and graft failure. A multifactorial strategy aimed at prevention of endothelial injury and graft failure should include improved surgical techniques, optimal preservation conditions, avoidance of nonphysiologic distension pressures, and use of specific pharmacologic agents as the primary form of intervention. The successful application of this strategy, and the development of newer and more efficacious strategies that may impact on long-term graft patency, can now be aided by assessment of the structural and functional integrity of bypass conduits using multiphoton imaging techniques.

(Ann Thorac Surg 2001;72:S2245–52)

The vascular endothelium is a hemocompatibile monolayer of mesenchymal cells forming a barrier between the circulating blood and etxravascular space, and is known to be a complex modulator of a variety of biological systems [1, 2]. The preservation of endothelial cell viability is vital for inhibiting early pathologic changes and the long-term patency of vascular grafts [1–7]. Restenosis of venous bypass grafts can occur as a healing and remodeling response to the initial tissue injury in a poorly regulated process. Despite the widespread use and superior patency of the internal mammary artery (IMA), the saphenous vein continues to be the most commonly used conduit for coronary artery bypass graft (CABG) and peripheral arterial surgery. The pathologic changes leading ultimately to vein graft occlusion and loss in vasomotor function are well documented [1–7]. Endothelial damage appears to be a major cause of graft failure. This injury may occur at the time of harvest, due to blunt surgical trauma and stretch [1, 4, 6], or to ischemia and superoxide free radical generation during prolonged ex vivo preservation, storage conditions, and distension or pressurization before anastomosis. Endothelial trauma is also caused by exposure to arterial circulation pressure and oxygenated blood [1, 8] graft insertion [9, 10], turbulent blood flow or local stasis from slow boundary layer flow [10].

This review elucidates the potential causes and adverse effects of intraoperative injury to the endothelium of an aorto-coronary bypass conduit, and underscores the relationships between intraoperative endothelial injury and the pathogenesis of early and late graft failure. It also introduces a novel method employing multiphoton fluorescent imaging for the intraopertaive assessment of aorto-coronary bypass graft endothelial function.

Pathogenesis of Endothelial Dysfunction in Aorto-Coronary Bypass Conduits

Stage 1: Thrombogenesis

Venous graft failure within 1 month after CABG surgery is thought to occur as a result of thrombogenesis [1–9, 11]. Early pathologic changes observed in autogenous saphenous vein grafts implanted as arterial autografts include acute thrombosis, denudation of endothelium, platelet and fibrin accumulation, cellular intimal subendothelial infiltrates, myointimal proliferation, smooth muscle necrosis, and inflammation [1–9].

Endothelial cells are known to be important mediators in regulating platelet anticoagulant as well as procoagulant processes and fibrinolytic functions. These properties of the endothelium facilitate circulation of blood; they also facilitate blood clotting and thrombogenesis following endothelial injury [6–11]. Damage to the endothelium or denudation of endothelial cells activates the intrinsic pathway of the coagulation cascade due to the exposure of basement membrane collagens, as shown in Figure 1 [1, 11]. Endothelial injury and inflammatory cytokines such as tumor necrosis factor (TNF), interleukin-1 (IL-1), and complement fragment 5a also activate the extrinsic coagulation processes by the induction of tissue factor that is constitutively expressed on the endothelial cells and the exposed subendothelial matrix [1, 4, 6, 11]. Both pathways activate clotting factor X, which

Presented at Monitoring and Improving Patient Safety During and Following Cardiac Surgery, San Diego, CA, May 5, 2001.

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Published by Elsevier Science Inc

0003-4975/01/$20.00
PII S0003-4975(01)03272-6

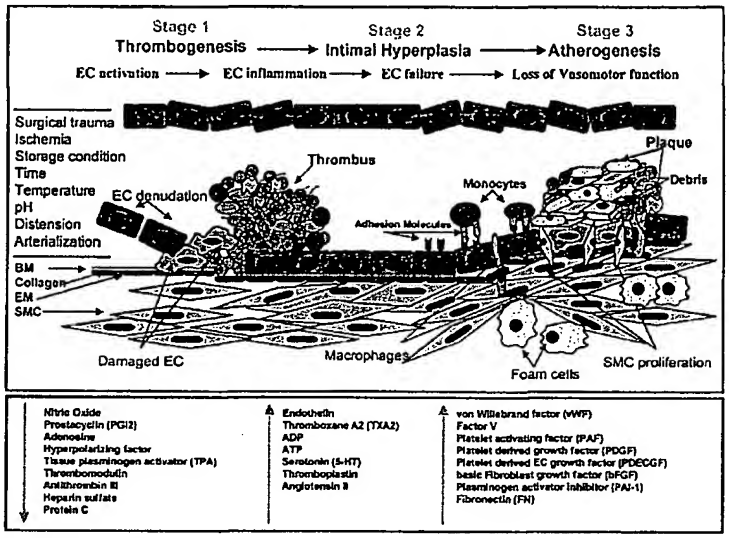

Fig 1. Stage 1 (<1 month after CABG surgery): explantation of saphenous vein leads to denudation and damage to the endothelium. Platelets (brown filled circles), *neutrophils* (pink filled squiggles [resembling "approximate" signs]), *monocytes* (green filled circles), *and fibrin* (light purple wavy lines) *are recruited on the exposed basement membrane* (BM) *and extracellular matrix* (EM), *resulting in decrease in release of anticoagulant and vasorelaxant factors* (red downward arrow) *and increased secretion of procoagulant and vasoconstrictor effectors* (red upward arrows), *leading to thrombogenesis, endothelial cell* (EC) *activation, and inflammation. Stage 2 (>1 month after surgery): activation, inflammation, and aggregation of EC, platelets, and recruitment of leukocytes induces intimal hyperplasia by the proliferation of smooth muscle cells* (SMC). *Stage 3 (generally >3 years after surgery): monocytes transformed into macrophages, migrate to the subendothelial layers, accumulate lipid particles, and become foam cells. Simultaneously, SMC migrate and proliferate into the lumen, entrapping foam cells, cellular debris* (brown wavy lines) *and recruited blood cells forming a plaque. Stages 1 and 2 result in EC failure, loss of vasomotor function, and ultimately stenosis and graft failure.*

converts prothrombin to thrombin and which, in turn, converts soluble fibrinogen to an insoluble meshwork of fibrin that is deposited on the damaged endothelium. Fibrin meshwork then traps blood cells, forming the thrombus [11]. Similarly, impaired secretion of prostacycline (PGI_2), nitric oxide, adenosine, and tissue plasminogen activator (tPA) by the damaged endothelial cells leads to platelet activation, recruitment, and aggregation, and deposition of fibrin on the damaged luminal surface. Aggregation of platelets, recruitment of monocytes and neutrophils, trapping of red blood cells, and fibrin meshwork lead to thrombus formation and embolism, as shown in Figure 1 [1–4, 6, 7, 9, 11]. This process is further accelerated by the interaction of platelets (mediated through Gp Ib and Gp IIb/IIIa complex, and CD41/CD61, $\alpha IIb\beta 3$ integrins) with high concentration of endothelium-derived von Willebrand factor, fibrinogen, and fibronectin deposited in the extracellular matrix [1, 4, 6, 11]. Further, activity of endothelial cell membrane-bound thrombomodulin is attenuated by loss of or damage to the endothelial cells. Thrombomodulins are known to exert antithrombotic effects by cleaving clotting factors VIIIa and Va by forming a complex with its cofactor protein S, thrombin, and the circulating anticoagulant protein C [4, 6, 11, 12].

Prostacyclin and nitric oxide secreted by the endothelium bring about the relaxation of smooth muscle by increasing platelet and smooth muscle cell cGMP. In contrast, adenosine released by the endothelial cells induces smooth muscle cell relaxation through its action by means of the adenosine receptor coupled G protein and adenylate cyclase, by modulating the cAMP levels in the conduits [13]. Endothelial injury impairs these biochemical pathways, leading to loss of vasomotor function and contributing significantly to graft occlusion [1, 5, 9]. Activated platelets recruited by damaged endothelium also secrete vasoactive substances such as adenosine diphosphate (ADP), adenosine triphosphate (ATP), serotonin (5-HT), thromboxane A_2 (TXA_2), and platelet activating factor. These substances potentiate vascoconstriction as well as further recruitment and activation of platelets, leading to accelerated thrombogenesis and graft failure, as shown in Figure 1 [1, 11]. Additionally, endothelium damaged or denuded saphenous veins are highly sensitive to the very potent endothelium-derived circulating endogenous vasoconstrictors, endothelin-1 [4–6], TXA_2, and angiotensin II [4, 5], which are all known to increase during CABG surgery [6, 7]. The increase in vascular tone mediated by these vasoconstrictors may lead to attenuated blood flow, stasis, and predisposition to thrombus formation in venous grafts. The differential rates of synthesis of PGI_2 and nitric oxide in arterial and vein grafts may possibly contribute to altered incidences of arteriosclerosis in these bypass grafts [1, 4, 6]. Therefore, greater production of endothelium-derived PGI_2 and nitric oxide in the internal mammary artery (IMA) compared with the saphenous vein results in lower incidence of arteriosclerosis in IMA grafts and, therefore, longer patency than with the saphenous vein grafts [1, 4, 6, 9]. The low fluid shear stress in venous grafts in contrast to arterial conduits also reduces the shear-

dependent release of tPA, nitric oxide, and PGI_2, further compounding this problem [1, 4, 6]. Endothelium also expresses and secretes heparin sulfate, which mediates an anticoagulant effect by binding to thrombin [4]. By potentiating antithrombin III (ATIII), heparin sulfate also prevents activation of thrombin and thus attenuates thrombogenesis. Poor expression of heparin sulfates in the media and internal elastic lamina of saphenous veins may contribute to thrombus formation in these grafts. This deficiency is further complicated by the endothelial and smooth muscle cell injury [6]. In contrast, significant expression of heparin sulfates in IMA may help prevent clot formation and thus maintain long-term patency of these grafts [6]. Therefore the vascular endothelial dysfunction plays a major role in the thrombogensis, which is thought to be the primary cause of vein graft occlusion and graft failure during the first month after CABG surgery [1, 4, 6, 9].

Stage 2: Intimal Hyperplasia

The 1-year patency of the saphenous vein graft is low relative to the that of the IMA, the occlusion rate in the saphenous vein being of 15% to 26% in the first year [1, 6, 7, 9]. Venous graft failures between 1 month and 1 year after CABG surgery are thought to occur as a result of intimal hyperplasia [1, 4, 6, 9]. Proliferation of smooth muscle cells and intimal hyperplasia is generally not observed with a normal endothelium in intact saphenous veins. However, nearly all arterialized vein grafts demonstrate a significant decrease in lumen size due to intimal thickening within 4 to 6 weeks after implantation [6, 10, 14]. Even though the decrease in lumen size may not contribute to stenosis in these early stages, the intimal hyperplasia is responsible nevertheless for the decrease in lumen size and forms the nucleation site for the later development of graft atheromas. Irrespective of whether they are arterial or vein grafts, neointimal hyperplasia in injured vessels demonstrate similar pathogenic sequelae. Injury or denudation of the endothelium stimulates the proliferation of smooth muscle, leading to the formation of thickened intima (Fig 1). This hyperplastic reaction is attributed to the release of various cytokines and growth factors such as platelet-derived growth factor (PDGF), basic fibrobalst growth factor (bFGF), platelet-derived endothelial cell growth factor (PD-ECGF), agiogenesis factor, and transforming growth factor β (TGF-β), by the activated platelets, injured endothelial cells, and injured smooth muscle cells in the intima and media [1, 4, 6]. In response to these growth factors and cytokines, smooth muscle cells in the media initially proliferate and then migrate to the intima, where they continue to undergo hyperplasia. Subsequent synthesis and deposition of extracellular matrix by activated smooth muscle cells, formation of neoendothelium over a layer of platelets, and fibrin adhering to the basement membrane result in a progressive increase in intimal fibrosis that eventually leads to obstruction of the lumen and to failure of the graft (Fig 1).

The considerable amount of endothelial damage observed in vein grafts before anastomosis and during the process of arterialization leads to greater secretion of growth factors and cytokines and, consequently, to an increase in intimal smooth muscle cell proliferation, stenosis, and failure of saphenous vein grafts. In contrast, internal mammary artery grafts, free of endothelial damage and already acclimated to the arterial environment, develop significantly less intimal hyperplasia and demonstrate greater long-term patency after aorto-coronary bypass surgery [1, 6, 9, 10]. Unlike arterial grafts, the "ischemia-reperfusion" cycle encountered by venous grafts may be an additional mechanism of injury and intimal hyperplasia in these conduits. Injury sustained by the endothelial cells not only results in a decrease in prostacyclin, nitric oxide, and adenosine synthesis, but also leads to the formation of superoxide radicals that directly promote smooth muscle cell proliferation [1, 4, 6]. This decrease in nitric oxide production is further compounded by the destruction of released nitric oxide by the superoxides, thus decreasing the endothelium dependent vasomotor function of the vein graft.

Dilation of the vein conduits in the arterial circuit due to pressure differences leads to an increase in vein diameter and, thus, to a decrease in blood velocity and shear stress [6, 10]. This results in an increased production of shear-regulated potent mitogens such as PDGF, bFGF, and endothelin-1, simultaneously decreasing the production of growth inhibitors such as nitric oxide and transforming growth factor β, thus shifting the equilibrium in favor of smooth muscle cell proliferation and intimal hyperplasia and further contributing to graft failure [1, 4, 6]. Unlike the saphenous vein, the IMA is not explanted; does not require ex vivo storage; and undergoes minimal processing and handling before implantation. As such, it offers less potential for endothelial injury [5].

Stage 3: rteriosclerosis

Attrition of veinous grafts and recurrence of ischemic symptoms beyond 1 year after CABG surgery is attributed to atherosclerotic changes. Chronic injury to the endothelium and the smooth muscle cells leads to ongoing recruitment of platelets, neutrophils, monocytes, macrophages, and lymphocytes at the site of injury and to resultant inflammation (Fig 1). Endothelial cell damage occurring at the time of saphenous vein grafting can be the principal event triggering both the development of late atherosclerotic changes and their subsequent adverse effects on cellular structure, endothelial function, and vasoreactivity [1, 5]. Endothelial cell growth and proliferation is contact-regulated [4]. These cells try to reestablish contact by stretching out and proliferating in the wounded region. However, because of chronic injury and loss in function, endothelial cells loose their capacity to replicate, leading to areas of subendothelial exposure. This allows the adhesion of platelets and inflammatory cells in these regions, which become activated and induce the smooth muscle cells to proliferate and to cover the denuded region, as shown in Figure 1 [4].

Nitric oxide, prostacyclin, and adenosine, apart from their vasorelaxation function, are also potent antiadhesive mediators that prevent neutrophil–endothelial adhesion under normal physiologic condition [4]. However, sublethal damage to the endothelial cells can not only cause the loss of these antiadhesive effector molecules, but can also cause induction and upregulation of leukocyte adhesion molecules on the endothelial cells; the latter attract neutrophils, monocytes, and macrophages to these areas (Fig 1). The activation of adherent leukocytes leads to the release of oxygen-derived free radicals, proteases, and cytokines such as TNF, IL-1, IL-6, and IL-8 and to further recruitment of inflammatory cells; these in turn perpetuate disruption of the subendothelium and induce smooth muscle cell proliferation [4, 6]. Tempo-

rally, atherosclerotic plaques develop by the transformation of lipid-filled macrophages into foam cells and encapsulation of proliferating smooth muscle cells [4, 6]. Atherosclerotic plaques progress by the accumulation of alternate layers of foam cells, smooth muscle cells, and necrotic debris, as shown in Figure 1 [4]. Vein graft atheromas, in contrast to native vessel atheromas, are found to be diffuse, concentric, and friable, with poorly developed fibrous cap and without calcification [6]. The plaques continue to grow and to encroach into the lumen, thereby impeding blood flow. In later stages, they may recruit thrombii or may rupture, leading to stenosis, graft failure, and myocardial infarction. In contrast to vein grafts, IMA grafts can resist the formation of atheromas [6]. Internal mammary artery conduits, therefore, tend to be superior in comparison to saphenous vein grafts because of the low incidence of thrombogenesis, intimal hyperplasia, and arteriosclerosis observed in these grafts. Also, IMA grafts are not explanted; therefore, sympathetic and parasympathetic control of IMA grafts may also play an important role in maintaining the patency of these conduits. In follow-up reports of patients evaluated 5 to 12 years after CABG surgery, the patencies confirmed by angiography were 95% for IMA versus 55% for saphenous vein grafts [1, 6]. These differences were more apparent when both conduits were used in the same patient [1].

Measuremnt of Endothelial Function

The intraoperative preservation of harvested saphenous veins before performance of coronary artery bypass grafting is a factor in the protection of the endothelial cells. Short-term preservation of free vascular grafts is a daily routine in coronary operations, in which 1 to 2 hours may elapse between harvesting and reperfusion. This interval may affect both the structure and function of the graft, depending upon the composition of the storage solution, the storage temperature, or the duration of ischemia before reimplantation [1, 4]. Because endothelial damage appears to be a major cause of graft failure, defining the time and nature of this injury is of prime importance. Currently available assays define vascular damage by measuring vasomotor function, including primarily static vascular cell culture, ring studies of sectioned vascular tissues in an organ bath, and animal models in which relaxation of precontracted tissues is measured [5, 15]. Collectively these techniques are limited by the lack of concomitant in situ assessment of the viability of the endothelium. Furthermore, they do not allow for simultaneous visualization of the structural integrity of endothelial and smooth muscle cells. They also do not allow the real-time measurement of the function of these cells in the form of calcium signaling or nitric oxide generation.

Until very recently, the intracellular events transpiring within endothelial cells in intact living vessels could not be evaluated directly because of the inability of conventional fluorescence microscopy to image deep into thick tissues. Although, tissue culture models of endothelial cells have been widely explored, these experimental models may provide only limited insight into the mechanisms that operate in tissues in situ. However, with the recent development of multiphoton fluorescence microscopy [16], these limitations have been overcome by allowing deeper penetration into the tissues using imaging technology through the use of the infrared region of the electromagnetic spectrum as an excitation source [16–18]. Therefore, optical sectioning and construction of three-dimensional images has become possible without physically dissecting the sample. Localization of multiphoton excitation to a limited volume at the focal plane minimizes photo-damage and out-of-plane fluorescence, thereby generating high-contrast, well-resolved images from deep within the tissues [16–18]. Multiphoton microscopy has been used in the three-dimensional functional imaging of diverse tissues such as cornea [19], human skin [18], mammalian kidney and brain [11], cardiac myocyte [20], and mammalian embryo [21]. We have successfully applied this imaging technology in real time to assess the structure and physiologic function of explanted, living, intact human saphenous veins preserved in the course of cardiac surgery [22, 23]. The next section of this article reviews the methodology and the lessons learned to date from this investigation.

Application of Multiphoton Microscopy to Intraoperative Assessment of Endothelial Function in Whole Aorto-Coronary Bypass Conduits

Structural Assessment

The structural and functional viability of vessel endothelium is measured with a fluorescence-based, supervitality, live–dead assay [24]. This assay measures the structural integrity of cells by allowing the membrane-permeable calcein AM ester dye to enter the cell and be transformed by the cellular esterases to produce green fluorescence in living cells, thus providing a measure of enzyme activity and cell viability. In contrast, the membrane impermeable ethidium homodimer enters compromised cells and intercalates with nucleic acids, giving red fluorescence in dead or damaged cells, demonstrating altered structural integrity of the cells. The high affinity for DNA and low membrane permeability of ethidium homodimer makes it an ideal indicator of dead cells [22–24]. This combination of calcein and ethidium homodimer has been applied as a viability assay in brain, sperm, and corneal tissue [24]. Calcein, alone, has been used as a marker of cell viability in cultured human saphenous vein endothelial cells [25]. The application of this two-color viability assay to the cells of whole segments of human saphenous vein has recently been reported [22, 23].

To estimate the penetration depth required to visualize the lumen and the endothelial cell layer in whole saphenous vein, vein segments freshly obtained from the operating room are divided into two pieces and cannulated. Both pieces are then labeled with calcein and ethidium homodimer dyes by way of the lumenal region. Using multiphoton microscopy we are able to image successfully deep into the saphenous vein, as shown in Figure 2 [22, 23]. The lumen, endothelial cell layer, and smooth muscle layer of the intimal region are identified at a depth of ~150 μm in these preparations. The endothelial and subsequent smooth muscle cell layers are clearly visible surrounding the lumen; the viability of these cell layers is indicated by the robust green cellular fluorescence (Fig 2A). The fluorescent signal of media and the adventitia was less intense, probably reflecting the short time of incubation with the fluorescent dyes, which must diffuse from the vein lumen to label the outer layers of the vessel. In one piece the endothelial cells in the lumen are intentionally damaged by short exposure to the detergent Triton-X100. As expected, considerable

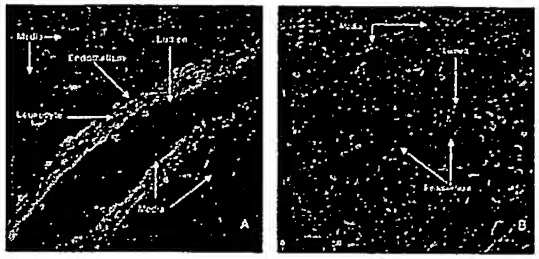

Fig 2. Cell viability assay in intact human saphenous vein. A freshly segmented vein was labeled with calcein and ethidium homodimer in Hank's balanced salt solution. (A) Green cellular fluorescence indicates cell viability. (B) Red nuclear fluorescence shows compromised or dead cells. The smooth muscle cell layer in the intimal region of the vein is identifiable at ~100 μm and the vein lumen becomes visible at ~150 μm. To differentiate between living and dead cells, the endothelial cells were made permeable with 0.1% triton before labeling as shown in part B. Figure 2 demonstrates the ability to identify the lumen and endothelium, as well as to differentiate between living and dead cells in an ex vivo, intact vein using multiphoton microscopy.

cell death, indicated by presence of red fluorescence, is observed in the clearly identifiable endothelial region of the lumen in the detergent-treated vein piece (Fig 2B). By using these protocols we consistently resolve the vein lumen up to a depth of 300 μm for structural and functional investigations [22, 23].

Functional Measurement

The functional viability of saphenous vein was also assessed by the ability of the endothelium to produce nitric oxide. The measurement of nitric oxide cannot easily be performed in biological tissues or fluids because of its rapid metabolism and low intracellular concentrations. Consequently, plasma nitric oxide concentration is usually determined by measuring both NO_2^- and NO_3^- concentrations, with limited application [26]. However, direct quantitation of nitric oxide synthesis in the lumenal endothelium of an intact vessel can bypass these limitations. Introduction of nitric oxide–specific fluorescent dyes such as diaminofluorescein has helped to overcome the drawbacks of other assays that have been used to measure nitric oxide synthesis [27]. This dye thus offers a unique approach to the examination of endothelial cell function in intact saphenous vein grafts [22, 23].

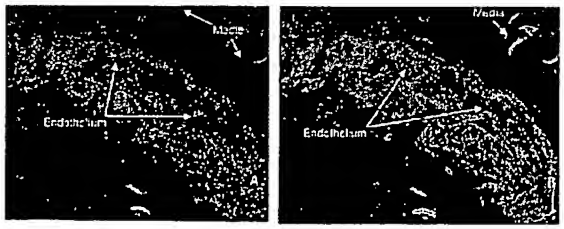

Fig 3. Real-time imaging of nitric oxide generated in saphenous vein segments using multiphoton microscopy. The vein segments were incubated with nitric oxide indicator dye DAF-2/DA in Hank's balanced salt solution for 1 hour at 37°C and mounted on the microscope stage. After identifying the vein lumen by XYZ scanning, microscope was slightly defocused allowing for larger endothelial area for quantification of nitric oxide. By this maneuver, a larger endothelial cell area was available for quantitation of nitric oxide. eNOS was activated with bradykinin (10 μmol/L) and the temporal increase in nitric oxide–mediated fluorescence was measured. (A) Representative image of eNOS activity before activation. (B) The same vein segment imaged 10 minutes after eNOS activation. A 2.5- to 3-fold increase in fluorescence due to the production of nitric oxide was observed.

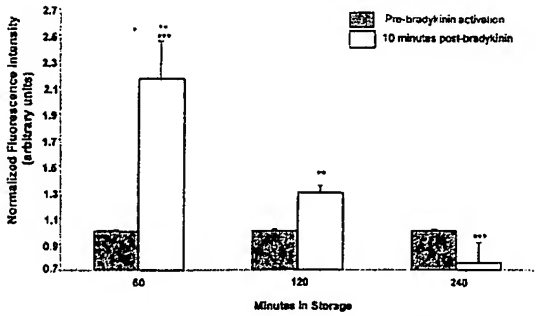

*Fig 4. Temporal changes in human saphenous vein eNOS activity. The vein segments were incubated with DAF-2/DA and processed for endothelial nitric oxide generation. The integrated DAF-2 fluorescence intensity of endothelial region of each vein stored at various time points was measured after 10 minutes of treatment with bradykinin (10 μmol/L) and normalized to the fluorescence intensity measured before the drug treatment. Six independent vein preparations were examined. Changes in fluorescence intensity between basal and bradykinin-treated vessels decreased significantly with time of storage and between various time points. Bars represent mean ± SEM. p = 0.003 by analysis of variance for 10 minutes after bradykinin treatment over time; * p = 0.026, prebradykinin versus postbradykinin at 60-minute value; ** p = 0.017, postbradykinin 60-minute versus 120-minute value; ***p = 0.002, postbradykinin 60-minute versus 240-minute value. (Reproduced from Ref. 22 by permission).*

The production of nitric oxide in intact saphenous vein was measured directly in real time by using a nitric oxide–specific, membrane-permeable diacetate form of fluorescent diaminofluorescein dye (DAF-2DA). This dye is cleaved by the endothelial esterases in living cells and is converted to its membrane-impermeable form, DAF-2. This DAF-2 dye, in the presence of molecular oxygen, combines with intracellularly generated nitric oxide to yield the brightly fluorescent triazolofluorescein derivative [27]. Temporal change in intracellular fluorescence is imaged and quantitated using multiphoton microscopy. Nitric oxide assays are performed under conditions that are encountered after the intraoperative harvesting and the short-term, ex vivo storage of the saphenous vein [22, 23]. To study endothelial function, vein segments harvested in course of cardiac surgery are loaded with DAF-2DA. Lumenal eNOS is functionally activated with bradykinin and the endothelial layers are identified by XYZ scanning. Figure 3 demonstrates a 2.5- to 3-fold increase in fluorescence in the endothelial region of the saphenous vein after bradykinin treatment. This increase in fluorescence, which is directly related to endothelial cell functionality, is quantitated by temporal integration of per pixel change in fluorescence in specific regions of the endothelium.

Effect of Storage Conditions on Harvested Human Saphenous Vein

hen the saphenous vein is harvested intraoperatively and stored in heparinized crystalloid solutions or blood, a rapid loss in the structural integrity of endothelial cells is observed, coupled with a decrease in the ability of

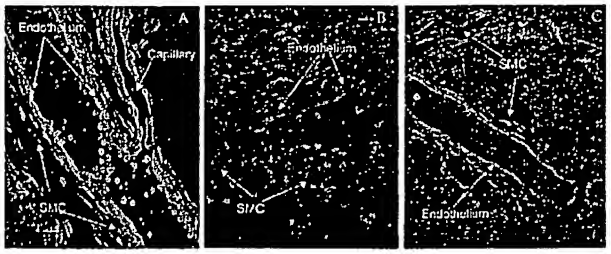

Fig 5. pH-Dependent viability of vein endothelium. Human saphenous vein segments were stored in Hank's balanced salt solution at various pH for 60 minutes at 21°C and were then labeled with calcein/ethidium homodimer. Green cellular fluorescence indicates cell viability and red nuclear fluorescence shows compromised or dead cells. (A) Cell viability was well preserved in veins stored at pH 7.4. (B) Significant cell death can be seen in vessels stored at pH 6. (C) Combination of living and dead cells are visible in veins stored at pH 8.0. SMC = *smooth muscle cells. (Reproduced from Ref. 22 by permission).*

endothelial cells to produce nitric oxide [23]. The deterioration in structural and functional viability of the vessel is dependent on the pH and time of storage [22], and also on the composition of the storage solutions [23]. During prolonged ex vivo storage of harvested saphenous veins, an increase in anaerobic metabolism is observed. As a result, lactic acid accumulates, with a significant increase in acidosis and a concomitant decrease in pH [23]. Our experiments document the detrimental effect of acidic storage conditions on the structural integrity of endothelial cells. Figure 4 shows the effects of varying pH of the preservation solution on endothelial and smooth muscle cell viability. Viability of all cell types is well maintained at physiologic pH levels of 7.3 to 7.4, as demonstrated by the green fluorescence (Fig 4A). In contrast, the viability of the cell decreases markedly at pH 6.0, indicated by the abundance of red fluorescence of the endothelial cell and smooth muscle layers (Fig 4B). At pH 8.0, an intermediate stage of viability is observed as shown by the mixed population of green and red fluorescence throughout all cell layers of the vein (Fig 4C). The adverse effects of nonphysiologic pH on endothelial cell viability are intuitive, however, there are no published data validating the effects of extreme pH on the endothelial cells in storage.

A time-dependent loss in endothelial structural viability is observed in stored saphenous vein [22]. Similarly, a temporal decay in the ability of endothelial cells to generate nitric oxide is also observed. As seen in Figure 5, the base line intensity remains stable over time. However, a steady and significant decrease in bradykinin-stimulated, eNOS-dependent nitric oxide generation is observed in the course of the preservation period. After 240 minutes of storage, the veins completely loose their ability to synthesize nitric oxide. Interestingly, in our study, the tonic activity of eNOS and nitric oxide production is not affected by the time in storage (Fig 5). It is the agonist-mediated activation of eNOS that degenerates over time. This observed decrease in eNOS activity, as well as the subsequent loss of the ability of the endothelial cell to generate nitric oxide, may adversely affect the vasoreactivity and long-term patency of the vein graft.

Studies with multiphoton imaging have shown a rapid deterioration of endothelial and smooth muscle cell function soon after vessel harvest, when stored in standard preservation solutions [23]. Therefore, a new physiologic storage solution called GALA (Hank's balanced salt solution modified with Glutathione, Ascorbic acid and L-Arginine) has recently been devised with the aim of maintaining the viability of cell structures and the integrity of key endothelial cell regulatory pathways, including nitric oxide synthesis [23]. The endothelium and smooth muscle cells of saphenous veins stored in GALA remain viable even after 24 hours of storage [23]. It is likely that storage in GALA solution during conduit harvesting may render an extended protective effect on the saphenous vein, thereby improving its long-term patency. However, clinical trials will be required to verify this assumption.

Effect of Distension on Human Saphenous Vein

Distension of vein grafts before anastomosis is a common practice in CABG surgery. This process allows the surgeon to check for the patency of the graft as well as for leakage. However, pressurization of the vessel above physiologic pressures with saline solutions causes a considerable amount of damage to the endothelium, intima, and media of the vessel [1]. By using calcein-ethidium homodimer assays and multiphoton microscopy we were able to observe the detrimental effects of distension on vessel structure. The convoluted viable endothelial regions of the freshly excised saphenous vein, identified by the green, living cell fluorescence (Fig 6A), were denuded and structurally damaged due to distension before anastomosis, apparent from the considerable amount of red fluorescence observed in the intima and media (Fig 6B). In contrast to a distended saphenous vein (>180 mm Hg pressure), a robust green fluorescence is observed in the endothelial region of IMA before anastomosis (Fig 6C), as the latter is not normally subjected to distension. It is interesting to note the differential arrangement of the convoluted endothelium in saphenous vein (Fig 6A) versus the flow-directed, linearly arranged endothelium in the IMA (Fig 6C).

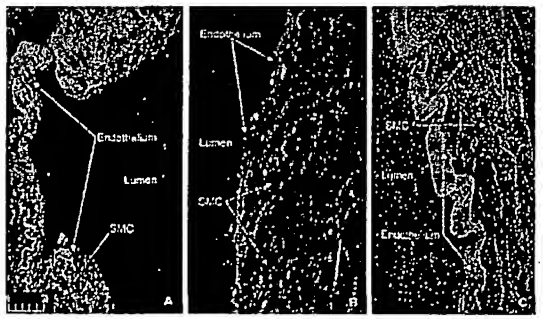

Fig 6. Distension-dependent changes in vessel viability. Saphenous vein and internal mammary artery segment were labeled with calcein/ethidium homodimer. Green cellular fluorescence indicates cell viability; red nuclear fluorescence shows compromised or dead cells. (A) Saphenous vein segment: living endothelium and intimal smooth muscle cells (SMC). (B) Distended saphenous vein segment: denuded and damaged endothelial and SMC. (C) Internal mammary artery before anastomosis: living endothelium and SMC regions. Note the differences in endothelial architecture of saphenous vein (A) and internal mammary artery (B).

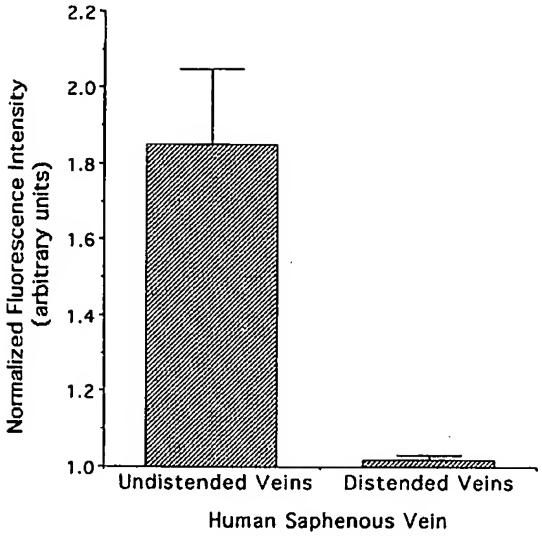

Fig 7. Distension mediated inactivation of eNOS activity in human saphenous veins. The control (undistended) and distended vein segments were incubated with DAF-2/DA and processed for endothelial nitric oxide generation. The integrated DAF-2 fluorescence intensity of endothelial region of each vein stored at various time points was measured after 10 minutes of treatment with bradykinin (10 μmol/L) and normalized to fluorescence intensity measured before drug treatment. Three independent vein preparations were examined. Increase in fluorescence intensity due to nitric oxide generation was significantly greater in undistended vein compared with distended vein ($p < 0.01$). Bars represent mean ± SEM.

Distension induced structural damage to the endothelium also translates into attenuation of its function, as shown in Figure 7. Injury to the endothelial cells results in severely impaired generation of nitric oxide in vessels that are dilated with the conventional saline and syringe distension procedure (Fig 7). Therefore, it is clear that using pressures above physiologic range for distension causes structural and functional abnormalities in both the endothelium and the smooth muscle cells of venous grafts.

Summary

Endothelial dysfunction is the primary determinant in the interrelated pathogenesis of thrombosis, intimal hyperplasia, and arteriosclerosis in aorto-coronary saphenous vein graft failure. The plethora of risk factors that can cause endothelial abnormalities and graft failure include (but are not restricted to) surgical trauma, ischemia, storage conditions, distension, and arterialization of venous grafts. Inherent deficiencies of the vein as a bypass conduit, structural and functional damage to the endothelium, and exposure of vein conduit to high oxygen tension and arterial pressure amplify the pathologic effects of risk factors. The compendium of these factors causes endothelial damage before transposition of the vein graft into the aorto-coronary circuit, and thus affects the long-term outcome of the saphenous vein graft. Multiphoton imaging can be successfully applied to elucidate the metabolic changes that take place during intraoperative storage of saphenous veins. This methodology can be used as a model to examine the effects of various storage conditions, agonists, antagonists, and other mediators of vein function. Intraoperative studies in which the endothelial structure and function were assessed by multiphoton microscopy have demonstrated marked endothelial dysfunction and loss of endothelial viability with current storage conditions and distension. These adverse effects, which might account for long-term graft failure, can be partly ameliorated by the use of the new preservative solution GALA and by avoiding distension of the aorto-coronary bypass conduits.

We thank Vaidya (Dr.) Ajit S. Kolatkar for sharing his knowledge of blood circulation and useful discussions. HST acknowledges Aditi Thatte for her encouragement and support. The editorial assistance of Mrs. Nancy Healey is greatly appreciated.

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DISCUSSION

I would like to congratulate Drs Thatte and Khuri on a very nice presentation. Their paper and presentation nicely describe the influence of endothelial cell viability and function on graft patency. Although there is little evidence that suggests that endothelial injury has much to do with short-term patency of grafts provided the anastomoses is technically sound and of sufficient caliber, there is significant evidence that, within several weeks postoperatively, the health of the vascular tissue (particularly the endothelium) can have a dramatic effect on the patency of bypass grafts. This is due, of course, to the production of nitric oxide, prostacyclin, and other endothelially derived vasodilators, and also to the inhibitory effect by these substances on platelet and neutrophil aggregation. These substances may have a dramatic effect on long-term patency.

I have several comments regarding the paper. First, a minor point. It was stated that adenosine was released from the endothelial cells. The vast majority of adenosine is released from the vascular smooth muscle and, probably more importantly, also from the myocardium. Second, it was mentioned that intimal hyperplasia is generally not observed with normal endothelium in intact saphenous veins. It is difficult to make this assertion, as endothelial function after vein harvesting probably is never normal, although it is suggested that this may be the case if one uses the GALA solution. A correlation between endothelial function and intimal hyperplasia has, to my knowledge, not been well demonstrated, and the development of intimal hyperplasia may likely be observed with or without the presence of functional endothelium in vein grafts. As mentioned in the presentation, one of the reasons that arterial grafts have a much higher patency than venous grafts is that the arterial endothelium is far more adherent to the basement membrane and the underlying vascular smooth muscle than the venous endothelium. In addition, the production of nitric oxide, prostacyclin, and other vasoactive metabolites occurs more in the arterial grafts than in the vein grafts. These antiadhesion substances decrease the development of platelet adhesion and thrombus formation. In the paper it was hypothesized that sympathetic and parasympathetic control of the IMA and other arterial grafts might play an important role in maintaining the patency of these conduits. Such a role, again, has not been clearly shown.

The authors demonstrated a new method for imaging arterial and venous bypass grafts. It may have considerable promise, especially in the development of new preservation solutions. Careful handling of the venous bypass conduits has a great impact on long-term patency that has truly been underestimated. It is easy to get the vein out and to distend it with about 400 pounds of pressure per square inch, but this can have very damaging effects on the endothelium and, consequently, on the long term patency. This should not be underestimated. As was mentioned in the presentation, there is good evidence that the addition of blood, albumin, or other preservative components can improve graft patency through preservation of the vascular endothelium. The addition of blood is potentially a very simple method to improve long term patency, especially in the venous grafts. Other novel preservative solutions have included newly developed polymers that can have a dramatic effect on endothelial preservation. This too is an underestimated and underused technique that has great promise for clinical use.

As the authors demonstrated, overdistention can have very harmful effects on veins and arterial grafts as well. However, some distention is necessary to ensure that there are no leaks and that the patient does not have to be brought back to the operating room because of bleeding the night after surgery. Distention causes activation of protein kinases and mitogen-activated protein (MAP) kinases, which initiate proliferative mechanisms and which, in addition, may be involved in the chemotactic pathways. Therefore, care should be taken not to overdistend the venous grafts.

Finally, attention should be given to protecting the endothelium, not only in the conduits but also in the native coronary arteries. The use of blood or magnesium supplementation in the crystalloid cardioplegia can inhibit calcium influx, and can also have a marked effect on preserving endothelial function in the native circulation. Therefore, these simple measures may decrease the incidence of early graft thrombosis due to reduced runoff. They may lessen the likelihood of postoperative spasm in the native circulation, and they may also decrease the incidence of intimal hyperplasia and late arteriosclerosis, not only in the arterial and vein grafts but also in the native circulation. It has been demonstrated in certain models that endothelial function can be recovered after several weeks. However, these studies were performed largely in the arterial circulation; whether the venous endothelium will recover is doubtful and has not been tested.

All these considerations underscore the importance of preserving the endothelium at the time of surgery. The effects of the above measures on long-term graft patency still need to be determined, but these are all avenues that we should pursue to improve the outcome after coronary bypass surgery.

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0003-4975/01/$20.00
PII S0003-4975(01)03283-0